AD_____

Award Number: W81XWH-10-1-0256

TITLE: Role of VAPB-MSP, a Novel EphA2 RTK Antagonist in Breast Cancer

PRINCIPAL INVESTIGATOR: Meghana Rao

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN 37232-2681

REPORT DATE: December 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
December 2012	Final Annual Summary	15 April 2009 – 31 December 2012

4. TITLE AND SUBTITLE
Role of VAPB-MSP, a Novel EphA2 RTK Antagonist in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0256

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Meghana Rao

E-Mail: meghanarao13@gmail.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University Medical Center
Department of Rheumatology
T 3219 Medical Center North
1161 21st Avenue South
Nashville, TN 37232-2681

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

VAPB (VAMP-associated protein B) is an endoplasmic reticulum protein that regulates multiple biological functions. VAPB protein expression is elevated in human breast cancers and correlates with poor prognosis. Despite aberrant expression of VAPB in breast cancer, its function in tumor cells is poorly understood. In this dissertation we demonstrate the causal role between VAPB and breast cancer, specifically in three areas (1) cell growth (2) invasion and (3) tumor growth *in vivo*.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	48	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

Page

INTRODUCTION

In our studies proposed herein, we demonstrate the causal role between VAPB and breast cancer, specifically in three areas (1) cell growth (2) invasion and (3) tumor growth *in vivo*. Our results show VAPB is important for breast tumor cell growth, affecting proliferation both in vitro and in mammary gland transplantation experiments. We also provide evidence that VAPB elevates the invasive potential of mammary epithelial cells. These phenotypes are, at least, partly mediated through enhanced AKT activation, a critical mitogenic signaling pathway in breast cancer. Additionally, we generated VAPB-deficient mice that overexpress the rat homologue of HER2 specifically in the mammary gland and scored spontaneously arising tumors for size and proliferation. Collectively, the genetic, functional and mechanistic analyses suggest an important function of VAPB in human breast cancer.

BODY

Statement of Work

Specific Aim 1: Define the function of secreted VAPB-MSP in tumor cells

Task 1: Establishing MMTV-Neu VAPB-knockdown cells rescued with VAPB-MSP or full-length VAPB, and MCF-10A cells overexpressing full-length VAPB or VAPB-MSP

Summary of Results:

Ectopic expression of VAPB-MSP in HER2-expressing breast tumor cells

To investigate whether the VAPB-MSP domain is critical for enhanced spheroid growth, we designed and cloned a VAPB-MSP construct (sMSP) into a pCLXSN (retroviral vector) and infected MMTV-Neu knockdown cells and MCF10A cells that overexpress HER2 (Figure 1).

Techniques Acquired: Lentivrial-mediated siRNA knockdown, Retroviral-mediated protein expression

Task 2: Assessing colony size, proliferation, apoptosis in above cell lines

Summary of Results:

VAPB promotes tumor spheroid growth by enhancing tumor cell proliferation

Because the VAPB is often overexpressed in human breast cancer [1-3], we sought to determine if elevated VAPB expression enhances tumor cell growth. MCF10A-HER2 cells, an immortalized mammary epithelial cell line with low level of endogenous VAPB, were transduced to express human VAPB protein (Figure 2A). Tumor spheroid growth was quantified in a three-dimensional culture. As previously reported, control MCF10A-HER2 cells formed large acinar-like structures with a filled lumen [4]. Expression of VAPB in MCF10A-HER2 cells led to larger, irregular structures with invasive protrusions and a significant increase of spheroid size relative to parental and vector controls (Figure 2B).

To determine whether increased tumor spheroid size is due to increased cell proliferation, the tumor spheroids were stained for proliferating cell nuclear antigen (PCNA), a cell proliferation marker. As shown in Figure 2C, VAPB expression elevated the number of PCNA-positive nuclei per spheroid, suggesting that VAPB regulates cell proliferation. Apoptosis, as measured by cleaved caspase 3 staining of 3D spheroid cultures, was not significantly changed (Figure 7A).

As an independent approach to determine whether VAPB is necessary for tumor cell growth, we knocked down VAPB in MMTV-Neu cells. These cells are derived from spontaneously arising mammary tumors from the *MMTV-Neu*

mouse model [5]. Stable expression of two independent shRNAs significantly reduced VAPB protein levels (Fig. 3A) but not the related protein VAPA (Figure 3C), suggesting that shRNAs specifically downregulated VAPB expression. In contrast to the morphology of vector control spheroids, which displayed disorganized and irregular structures with protrusions, knockdown of VAPB resulted in much smaller and more compact tumor spheroids (Figure 4A). Since one of the VAPB shRNA sequences (KD #1) targeted the 3′ UTR, we were able to re-express full-length human VAPB protein (Figure 3B). Re-expression of VAPB restored tumor spheroid size and irregular morphology, demonstrating that phenotypes induced by shRNAs are VAPB specific and not due to off-target effects (Figure 4A). Knockdown of VAPB significantly reduced proliferation in spheroid cultures as quantified by PCNA staining (Figure 4B), without affecting apoptosis as measured by cleaved caspase 3 staining of 3D spheroid cultures (Figure 7B). Together, these data indicate that VAPB regulates tumor spheroid size by increased tumor cell proliferation.

VAPB-MSP is not sufficient to enhance VAPB-mediated spheroid growth

In order to investigate the function of VAPB and sMSP in tumor cells, we examined the effects of altering VAPB or sMSP expression in breast cancer cell culture models. First, we examined whether VAPB or sMSP is necessary for tumor cell growth in MMTV-Neu tumor cells. As shown in Figure 5A, expression of sMSP in VAPB deficient MMTV-Neu tumor cells was not sufficient to rescue spheroid growth. Analysis by confocal microscopy revealed that VAPB knockdown cells also formed smaller, more organized acini (Figure 5B). Similar results were found in MCF10A-HER2 cells. Restoration of full-length human VAPB but not sMSP enhanced spheroid size and irregular morphology (Figure 6). These data suggest that sMSP is not sufficient to rescue VAPB deficiency. Because the sMSP did not have significant effects on spheroid growth, we did not access proliferation and apoptosis as measured by PCNA and cleaved caspase-3 respectively.

Techniques Acquired: 3D-Matrigel culture, proliferation and apoptosis assays, confocal microscopy analysis of immunofluorescent images.

Task 3. Orthotopic transplantation of above cell lines into mammary gland of female syngeneic mice

Summary of Results:

VAPB promotes tumor growth in an orthotopic mammary tumor model

Next, we investigated the role of VAPB in tumor growth *in vivo* in a mammary tumor orthotopic transplantation model [6]. One million *MMTV-Neu* control or VAPB knockdown cells were injected into cleared mammary gland fat pads of FVB recipient female mice. VAPB knockdown tumor cells failed to form

tumors or formed very small, non-palpable tumors at five weeks post-transplantation, compared to parental or vector controls (Figure 8A). While parental or vector control tumors display densely packed tumor cells, VAPB knockdown tumors exhibit a reduced mammary tumor cell content (Figure 8B). To examine changes within the tumor epithelium, we assessed tumor proliferation and apoptosis in tissue sections by staining for Ki67 and cleaved caspase-3, respectively. We observed a significant decrease in tumor cells with Ki67 nuclear staining (Figure 8C). Whereas cleaved caspase-3 levels were unaffected (Figure 7C). These data suggest that loss of VAPB inhibits HER2-initiated mammary tumor proliferation *in vivo*.

VAPB deficient mice exhibit normal mammary gland architecture.

Our own studies revealed that VAPB, but not secreted MSP promotes tumor cell proliferation in cell culture (Figures 2-5). However whether the phenotypes induced by VAPB are reproducible in an animal model of breast cancer remain unknown. Therefore we generated (inGenious Targeting Laboratory) VAPB KO mice in order to test the function of VAPB in tumor cell growth *in vivo.* The targeting strategy and confirmation of genotype by southern blot are shown in Figure 9A and 9B. After backcrossing mice into the FVB background, we confirmed VAPB deficiency by western analysis. As expected VAPB protein was undetectable from whole mammary gland lysates from VAPB KO mice when compared with littermate WT controls (Figure 10A).

To determine whether VAPB deletion affects mammary gland architecture, we performed whole mount analysis of WT and VAPB KO mammary glands at 8 weeks of age. We observed no gross morphological changes in the outgrowth of epithelial cells into the fat pad (Figure 10B) and in number of epithelial branch points as quantified in Figure 10C. These results suggest that VAPB deficiency is not critical to mammary gland development under these conditions.

Reduced tumor number in MMTV/NeuT VAPB KO mice

To investigate the influence of VAPB expression on mammary tumor formation, we crossed VAPB knockout mice (FVB background) with MMTV-NeuT transgenic mice as diagrammed in Figure 9C. In this model, MMTV-LTR drives the expression *Neu*, the rat homolog of ErbB2 (HER2) along containing an activating mutation, specifically in the mammary gland. In this model, virgin, female mice develop tumors between 4-5 months of age [7]. At five months, tumors that were harvested from MMTV-NeuT-positive, VAPB WT or KO mice were enumerated and scored for size. Not only do we see a significant decrease in the number of tumors formed per mouse (Figure 11A), we also observed a reduction in overall tumor burden in VAPB KO mice relative to WT controls (Figure 11B). Western analysis of tumor lysates show that VAPB depletion in KO tumors, however protein expression levels of ErbB2 were not affected by VAPB deficiency (Figure 11B). Our *in vivo* results are consistent with our observations that VAPB deficiency also reduces spheroid growth (Figure 4A) in cell culture.

Techniques Acquired: Orthotopic transplantation of breast cancer cells into mammary glands of recipient mice, mouse colony maintenance, mouse tumor model crosses, geneotyping, tumor palpation, and histological analysis of breast cancer samples, including routine histology techniques.

Specific Aim 2: Investigate the mechanism of VAPB-MSP in regulation of Ras/MAPK signaling.

Task 1: Investigate if VAPB-MSP binds to EphA2

Summary of Results:

VAPB-MSP co-localizes with EphA2 in MMTV-Neu tumor cells

Purified Maltose Binding Protein (MBP)-MSP or MBP control was incubated with MMTV-Neu VAPB knockdown cells that lack sMSP, but have endogenous expression of EphA2. Under non-permeabilizing conditions, we observed co-localization of MBP-MSP and EphA2 (yellow) as determined by confocal analysis (Figure 12)

Techniques Acquired: Protein purification, immunofluorescense analysis

Task 2. Investigate if VABP-MSP binding results in increased Ras and MAPK activity

Purified VAPB-MSP fails to activate the ERK pathway

The ERK pathway has emerged as one of the primary signaling pathways mediating cellular responses with Eph receptors. There is much evidence to support the role of ephrin-A1 in inhibition of ERK activation in tumor cells. Therefore we tested whether VAPB-MSP can compete with ephrinA1-induced ERK inhibition. Previous studies have shown that MSP can antagonize Eph receptors and activate ERK in *C. elegans* [8,9]. As shown in Figure 13, ephrinA1 reduced phospho-ERK and phospho-EphA2 levels. However the purified Maltose binding protein (MBP)-MSP did not appear to compete with ephrinA1 to increase ERK activation.

Full length VAPB elevated AKT activity

To understand the mechanisms through which VAPB enhances proliferation, we investigated potential links between VAPB and signaling molecules relevant to tumor growth. Because ERK and AKT signaling pathways are two major players in regulating cell proliferation in mammary tumor cells, we assessed their activities by western blot analysis. MCF10A-HER2 or VAPB knockdown MMTV-Neu cells carrying either wild-type VAPB expression construct or the control vector were serum-starved overnight and stimulated with EGF at

the indicated time points. In response to EGF stimulation, there is a rapid increase in phosphorylation of AKT at both Thr308 and Ser473 residues, indicating activation of its kinase activity. The phospho-AKT levels were significantly increased in MCF10A-HER2 cells overexpressing VAPB than those carrying the control vector (Figure 14A). Re-expression of VAPB in knockdown cells restored AKT phosphorylation to control levels (Figure 14B). In contrast, ERK phosphorylation levels were not changed between two cell populations (Figure 14). These results indicate that AKT may play a key role in regulating cell growth in these cells.

To test if VAPB-mediated spheroid growth is dependent on AKT phosphorylation, cells were treated with an allosteric AKT inhibitor [10]. AKT inhibition significantly reduced spheroid growth of VAPB expressing cells (Figure 15A and 15C). As expected the AKT inhibitor reduced phosphorylated AKT, while phospho-ERK was unaffected (Figure 15B and 15D). Although these results do not rule out the contribution of other signaling pathways, our findings suggest that VAPB-induced proliferation is mediated, at least in part, through activation of the AKT pathway.

Techniques Acquired: Immunoblot analysis, inhibitor assays

Task 3. Determine the effects of VABP-MSP on the ability of EphA2 receptor to recruit RasGAP or Grb2/Shc.

This task was not completed because there was not enough rationale to move forward with this experiment. Our analysis of ERK and AKT signaling pathways demonstrate increased AKT activation with VAPB expression, rather than ERK. Since RasGAP and Grb2/Shc are upstream activators of ERK, it would be more reasonable to investigate molecules in the PI3K pathway in future studies.

Specific Aim 3: Investigate the role of VAPB-MSP in regulation of cell-cell adhesion.

Task 1: Assess E-cadherin localization in MCF10A and MCF10A/Her2 cells overexpressing VAPB-MSP

Summary of Results:

VAPB alters the morphology of MCF10A-HER2 acini.

While we demonstrated that ectopic expression of VAPB in MCF10A-HER2 cells increased acini growth in 3-dimensional culture (Figure 2), we also observed gross morphological changes in VAPB-expressing acini. Consistent with previous reports, parental and vector control MCF10A-HER2 cells formed non-invasive acini structures (Figure 16A) and intact basement membrane structures as seen with laminin V immunofluorescence staining (Figure 16B)

[11,12]. However with the expression of VAPB, we observed an increase in invasive growth into the surrounding matrix as quantified in Figure 15A. Further analysis of the VAPB-mediated invasive acini growth with confocal microscopy revealed a strong presence of E-cadherin at cell junctions but weak laminin V staining at the area of invasive growth into the matrix (white arrow) (Figure 16B).

To address the observations seen in Figure 16B, we examined the effects of VAPB expression on basement membrane integrity by monitoring laminin V deposition. MCF10A cells normally deposit laminin V at the basal surface of acini by day five [4], creating a continuous ring that surrounds each structure. Upon ectopic expression of VAPB in MCF10A-HER2 cells we observed a significant decrease in laminin V circumscribing each acini as analyzed by confocal microscopy at day nine (Figure 17A). At day 5, both vector and VAPB-expressing cells have a continuous laminin V layer surrounding the acini, suggesting that laminin V is sufficiently produced in early stages of acinar growth. The results at day five and nine are quantified in the lower panel (Figure 17B). These data support the idea that VAPB is an important factor that promotes the irregularity and invasive behavior of HER2-driven acini growth.

Techniques Acquired: 3D-immunofluroescense assays

Task 2. Investigate epithelial-to-mesenchymal (EMT) transition in cells overexpressing VAPB-MSP

Summary of Results:

VAPB expression elevates the invasive potential of MCF10A-HER2 cells

A critical step in the metastatic cascade is the acquisition of invasive characteristics such as increased tumor cell motility and the ability to break down ECM components [13]. Therefore, we tested whether VAPB allowed for these characteristics in MCF10A-HER2 cells, using Transwell (Boyden chamber) assays. As seen in Figure 18A, VAPB expression significantly increased MCF10A-HER2 invasion through the Matrigel-coated Transwell filters in response to EGF (Figure 18A), demonstrating that VAPB imparts to MCF10A-HER2 cells the ability to breakdown the Matrigel layer, prior to migration through the membrane pores. However the results presented in Figure 18B suggest that VAPB does not affect cell motility because the differences seen between vector and VAPB-expressing are diminished when using uncoated Transwell filters, which tests for directional cell migration. Thus, it is likely that VAPB enhances the invasive capabilities of MCF10A-HER2 cells at the level of ECM breakdown, rather than increasing cell motility.

Based on our earlier studies, we found that VAPB expression increases tumor cell proliferation through enhanced AKT activation (Figure 14 and Figure 15). In addition to proliferation, invasion is also a process modulated by the AKT pathway [14]. Therefore, we used the same allosteric AKT inhibitor as in Figure 15, to test whether VAPB-induced invasion is dependent on AKT. Indeed, we find

that pharmacologic inhibition of AKT significantly reduced invasion in VAPB-expressing cells (Figure 18C).

Interestingly we observe no significant difference in EGF-induced cell migration between vector and VAPB-expressing cells, suggesting that VAPB has little effect on cell motility. While it is tempting to speculate that VAPB increases invasive potential by inducing epithelial-to-mesenchymal transition (EMT), a process by which polarized cells gain motility, characterized by loss of E-cadherin expression [15], our data show clear junction staining of E-cadherin with or without VAPB expression. This suggests that downregulation of E-cadherin may not be the primary mechanism promoting invasion in the context of VAPB. However, a more thorough analysis of EMT markers such as Vimentin, Snail and Twist is needed [15].

Techniques Acquired: Transwell invasion assays (Boyden chamber)

Task 3. Analyze the role of VAPB-MSP in EphA2 & Arf6 mediated E-Cadherin localization

Summary of Results:

The AFR6-GTPase is a known regulator of endosome traffic a key modulator of cadherin movement along the endosomal pathway. Our preliminary data suggests shows a modest increase in ARF6-GTP in VAPB expressing MCF10A-HER2 cells. However our 3D culture analysis shows that E-cadherin staining is quite strong whereas laminin V staining is diminished, suggesting that ARF6 may not be the primary mechanism for increased invasiveness.

Another potential mechanism that warrants further investigation is the role of matrix metalloproteinases (MMPs). MMPs are a family of proteins that remodel the ECM through proteolytic degradation its components, including collagens and laminin. MCF10A-HER2 cells secrete specific MMPs such as MMP-2 and MMP-9 that break down ECM components and facilitate invasion [16]. Interestingly it has been shown that AKT activation can increase the expression and activity of both MMP-2 and MMP-9, leading to enhanced invasive capabilities [17-19]. Therefore, future studies are needed to explore the possibility that the invasive phenotypes observed with VAPB expression are dependent on MMPs.

While the potential involvement of MMPs suggests that VAPB promotes the breakdown the basement membrane, it is equally likely that laminin V is being mis-localized. This alternative hypothesis is supported by the fact that there are centrally-located cells with cytoplasmic localization of laminin V in VAPB-expressing structures (Figure 15B and 16A). This is also consistent with the fact that VAPB has reported functions in vesicle trafficking [Reviewed in [20]].

KEY RESEARCH ACCOMPLISHMENTS

- VAPB is highly expressed in human breast cancer clinical samples and human-derived tumor cell lines
- High VAPB expression correlates with poor patient outcome as determined from two independent microarray datasets
- VAPB enhances breast tumor growth by modulation of AKT activity
- Major Sperm Protein (MSP) domain of VAPB is not sufficient to promote breast tumor growth
- VAPB elevates the invasive potential of MCF10A-HER2 cells
- VAPB promotes breast tumor growth by modulating the protein secretory pathway
- VAPB-deficient mouse models of breast cancer have reduced tumor number

REPORTABLE OUTCOMES

Manuscripts and Poster Presentations

Rao M., Song W., Jiang A., Shyr Y., Lev S., Greenstein D., Brantley-Sieders D.M., Chen J. VAMP-associated protein B (VAPB) promotes breast tumor growth by modulation of Akt activity (2012) ***PLoS ONE*** 7(10): e46281.

European Molecular Biology Organization (EMBO)-General Meeting - *Vienna, Austria* (2011) – Poster *"VAPB drives proliferation and invasion of breast cancer cells through regulation of critical vesicle trafficking events"*

Gordon Research Conference–Mammary Gland Biology - *Newport, Rhode Island* (2009) -- Poster *"The Role of VAPB in Breast Cancer"*

Degrees

Ph.D. Cancer Biology (GPA 3.7/4.0) August 2007 – December 2012
Thesis: The Role of VAMP-associated protein B in breast cancer
http://etd.library.vanderbilt.edu/available/etd-11292012-144810/

Advisor: Dr. Jin Chen

Cell lines and animal models

MMTV-Neu VAPB knockdown
MCF10A-HER2 VAPB
MCF10A-HER2 VAPB-sMSP
MCF10A-HER2 ΔMSP
MCF10A-HER2 ΔCC

VAPB Knockout mice (FVB background)
MMTV-NeuT/ VAPB knockout mice (FVB background)

CONCLUSIONS

We recently observed that high expression VAPB in clinical breast cancer samples significantly correlated with reduced overall and relapse-free survival. Other laboratories have also reported similar results. A genome–wide microarray analysis of 50 human breast cancer cell lines and 145 clinical specimens revealed that VAPB is often amplified or overexpressed in breast cancer, and functions in metastasis initiation. VAPB is a member of the VAP family proteins that interact with a large number of intracellular proteins and known VAP functions include vesicle trafficking, lipid metabolism and transfer, and the unfolded protein response (UPR). In addition to VAPB's intracellular function, it is cleaved and secreted, acting as a ligand for Eph receptor tyrosine kinases. Despite VAPB's clinical relevance and its role in diverse cellular processes, the precise function of VAPB in breast cancer was poorly understood. Our work presented herein has advanced our understanding of VAPB in breast tumor cell proliferation and invasion.

Many breast cancers depend on the hyperactivation of AKT-PI3K for growth, evasion of apoptosis, and invasion [21,22]. We provide evidence that VAPB regulates breast tumor cell proliferation and invasion through AKT activation. Overexpression of VAPB in MCF10A-HER2 cells enhances phosphorylation of AKT. In contrast, knockdown of VAPB in MMTV-Neu tumor cells diminishes pAKT levels. Moreover, pharmacological inhibition of AKT significantly reduced three-dimensional spheroid growth induced by VAPB and invasion through Transwell filters. Although these results do not rule out the contribution of other signaling pathways that modulate cell proliferation, our evidence suggests that VAPB induces growth and invasion through AKT activation.

Future Directions

While our work implicates two mechanisms by which VAPB promotes breast tumor growth or invasion, further investigation is required to reveal VAPB's full potential in cancer. We are confident that continued work in this field of study will provide novel insights into breast cancer progression. We attempt to speculate on a few possibilities below.

How does VAPB modulate AKT activity?

Although we demonstrate that VAPB enhances breast tumor cell proliferation and invasion, at least in-part, through the AKT pathway, the exact mechanism by which this happens remains unknown. There are several steps leading to AKT phosphorylation, starting with phosphoinositide 3-kinase (PI3K) activation upon recruitment to autophosphorylated, ligand-activated receptor tyrosine kinases (RTKs) [22]. PI3K is a lipid kinase that catalyzes the synthesis of membrane phosphatidylinositol-3,4,5-P_3 (PIP3) from phosphatidylinositol-4,5-P_2 (PIP2). The generation of PIP3 on the inner leaflet of the plasma membrane

recruits AKT by direct interaction with its PH domain [22]. At the membrane, another PH-domain-containing serine/threonine kinase named PDK1 phosphorylates AKT on Thr308 [23] and while mTORC2 mediates phosphorylation of Ser473 [24].

Given the effects of VAPB on breast tumor cell proliferation and invasion is dependent on AKT activation, it would be interesting to examine how VAPB modulates upstream activators of AKT, such as PI3K, both at the level of recruitment to RTKs and PI3K activity. In this study it is also noteworthy that VAPB interacts with Arf1, a small, Ras-family GTP-ase. Interestingly, Arf1 has been implicated in the recruitment of the p85 subunit of PI3K to EGFR in breast cancer cells [25]. This raises the possibility that the VAPB/ Arf1 interaction enhances PI3K activity by promoting recruitment of its p85 subunit.

On the other hand, if VAPB does not affect AKT activation at the level of PI3K, it is possible that VAPB alters the availability of PI3K's intracellular substrate PIP2 at the plasma membrane. We can speculate on this idea because we, and others, have found that VAPB interacts with Nir2 (PITPB2), a phosphoinositol transfer protein [Table 3; [26,27]]. These proteins facilitate the transfer of phosphoinositols between various cell membrane interfaces. Nir2 has been proposed to target PIs from the ER to *trans*-Golgi PI4-kinases [28] ultimately generating PtdIns(4)*P*s at the Golgi. However, recent studies indicate that the majority of PtdIns(4)*P* resides in the plasma membrane [29], which is then converted to PIP2 by phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) [30].It is tempting to speculate that VAPB promotes AKT by elevating levels of PIP2 substrate for PI3K, perhaps through localized targeting of phosphoinositide (PIs) intermediates by Nir2.

Does VAPB relieve ER stress during tumor proliferation?

Proliferative tumor cells often hijack various cellular processes in order to support growth, exerting stress on organelles such as the ER which is the site of protein folding and secretion, lipid biosynthesis and calcium homeostasis [31]. Tumor cells face extracellular stresses such as hypoxia, nutrient deprivation or growth constraints by the extracellular matrix (ECM) as well as intracellular stresses such as high protein demand for growth factors and receptors [31]. Such harsh conditions activate the UPR through one or all three receptors (IRE1, ATF6 and PERK).

Evidence has emerged that VAPB activates the IRE1-XBP1 arm of the UPR [32]. Furthermore sustained IRE1-XBP1 activation has been shown to help cells enhance proliferation and evade cell death [33,34]. Interestingly the IRE1-XBP1 pathway has been implicated in breast cancer such that XBP1 expression is upregulated in certain breast cancers and correlates with poor prognosis in patients [35,36]. Additionally, *in vitro* studies have demonstrated that XBP1 confers resistance to apoptosis in breast cancer cell lines [37]. A role for the IRE1-XBP1 pathway in breast cancer is further supported by *in vivo* bioluminescence imaging of breast tumor cells expressing a XBP1-luciferase

reporter in which luciferase is expressed only when XBP1 is spliced by activated IRE1 [31,38], which also provides a useful mouse model to address the role of IRE1-XBP1 in VAPB-mediated breast tumor proliferation and invasion.

VAPB's therapeutic potential in breast cancer?

Our data presented thus far demonstrate that VAPB functions to increase breast tumor cell and invasion through enhanced AKT activity. This pathway is commonly dysregulated in breast cancer and often promotes resistance to current therapies [21], such as those that target HER2. Treatment of VAPB-expressing cells with two different PI3K-AKT inhibitors reduced spheroid size to a greater extent than non-VAPB-expressing cells. This suggests that VAPB is more dependent on AKT signaling and may increase sensitivity to AKT inhibitor-based therapies. Another step to determine the therapeutic potential of targeting AKT in VAPB-expressing cancers is to correlate high VAPB expression with elevated AKT activity in human breast tumor samples. The results from such studies would help stratify patient populations into those that would benefit most from AKT-targeted therapies.

Furthermore, our observations that high VAPB expression correlates with poor prognosis and the ability to detect its secreted form (MSP) in human serum [39] suggests that VAPB can be used as a potential biomarker in breast cancer diagnostics, both in tumor tissue and in serum. Despite numerous advances in mechanism-based therapies, breast cancer morbidity rates remain high. Therefore, it is imperative that alternate cellular processes, such as those modulated by VAPB, be further investigated as potential targets for breast cancer chemotherapy.

REFERENCES

1. Arriola E, Marchio C, Tan DS, Drury SC, Lambros MB, et al. (2008) Genomic analysis of the HER2/TOP2A amplicon in breast cancer and breast cancer cell lines. Lab Invest 88: 491-503.
2. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, et al. (2006) Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 10: 529-541.
3. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, et al. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515-527.
4. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256-268.
5. Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, et al. (2003) Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. Mol Cell Biol 23: 8691-8703.
6. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, et al. (2008) The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. J Clin Invest 118: 64-78.
7. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 54: 105-115.
8. Miller MA, Nguyen VQ, Lee MH, Kosinski M, Schedl T, et al. (2001) A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. Science 291: 2144-2147.
9. Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D (2003) An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in Caenorhabditis elegans. Genes Dev 17: 187-200.
10. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, et al. (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. Bioorg Med Chem Lett 15: 761-764.
11. Lu J, Guo H, Treekitkarnmongkol W, Li P, Zhang J, et al. (2009) 14-3-3zeta Cooperates with ErbB2 to promote ductal carcinoma in situ progression to invasive breast cancer by inducing epithelial-mesenchymal transition. Cancer Cell 16: 195-207.
12. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol 3: 785-792.
13. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674.
14. Chin YR, Toker A (2009) Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer. Cell Signal 21: 470-476.

15. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2: 442-454.
16. McCawley LJ, Matrisian LM (2000) Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today 6: 149-156.
17. Jin EJ, Park KS, Bang OS, Kang SS (2007) Akt signaling regulates actin organization via modulation of MMP-2 activity during chondrogenesis of chick wing limb bud mesenchymal cells. J Cell Biochem 102: 252-261.
18. Kim IY, Yong HY, Kang KW, Moon A (2009) Overexpression of ErbB2 induces invasion of MCF10A human breast epithelial cells via MMP-9. Cancer Lett 275: 227-233.
19. Kwiatkowska A, Kijewska M, Lipko M, Hibner U, Kaminska B (2011) Downregulation of Akt and FAK phosphorylation reduces invasion of glioblastoma cells by impairment of MT1-MMP shuttling to lamellipodia and downregulates MMPs expression. Biochim Biophys Acta 1813: 655-667.
20. Lev S, Ben Halevy D, Peretti D, Dahan N (2008) The VAP protein family: from cellular functions to motor neuron disease. Trends Cell Biol 18: 282-290.
21. Miller TW, Rexer BN, Garrett JT, Arteaga CL (2011) Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. Breast Cancer Res 13: 224.
22. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489-501.
23. Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, et al. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 277: 567-570.
24. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098-1101.
25. Boulay PL, Cotton M, Melancon P, Claing A (2008) ADP-ribosylation factor 1 controls the activation of the phosphatidylinositol 3-kinase pathway to regulate epidermal growth factor-dependent growth and migration of breast cancer cells. J Biol Chem 283: 36425-36434.
26. Litvak V, Dahan N, Ramachandran S, Sabanay H, Lev S (2005) Maintenance of the diacylglycerol level in the Golgi apparatus by the Nir2 protein is critical for Golgi secretory function. Nat Cell Biol 7: 225-234.
27. Peretti D, Dahan N, Shimoni E, Hirschberg K, Lev S (2008) Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport. Mol Biol Cell 19: 3871-3884.
28. Schaaf G, Ortlund EA, Tyeryar KR, Mousley CJ, Ile KE, et al. (2008) Functional anatomy of phospholipid binding and regulation of phosphoinositide homeostasis by proteins of the sec14 superfamily. Mol Cell 29: 191-206.

29. Hammond GR, Schiavo G, Irvine RF (2009) Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P(2). Biochem J 422: 23-35.
30. Fruman DA, Meyers RE, Cantley LC (1998) Phosphoinositide kinases. Annu Rev Biochem 67: 481-507.
31. Tsai YC, Weissman AM (2010) The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer. Genes Cancer 1: 764-778.
32. Kanekura K, Nishimoto I, Aiso S, Matsuoka M (2006) Characterization of amyotrophic lateral sclerosis-linked P56S mutation of vesicle-associated membrane protein-associated protein B (VAPB/ALS8). J Biol Chem 281: 30223-30233.
33. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, et al. (2007) IRE1 signaling affects cell fate during the unfolded protein response. Science 318: 944-949.
34. Lin JH, Li H, Zhang Y, Ron D, Walter P (2009) Divergent effects of PERK and IRE1 signaling on cell viability. PLoS One 4: e4170.
35. Davies MP, Barraclough DL, Stewart C, Joyce KA, Eccles RM, et al. (2008) Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. Int J Cancer 123: 85-88.
36. Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, et al. (2003) Upregulation and overexpression of human X-box binding protein 1 (hXBP-1) gene in primary breast cancers. Breast Cancer 10: 301-306.
37. Gomez BP, Riggins RB, Shajahan AN, Klimach U, Wang A, et al. (2007) Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. FASEB J 21: 4013-4027.
38. Spiotto MT, Banh A, Papandreou I, Cao H, Galvez MG, et al. (2010) Imaging the unfolded protein response in primary tumors reveals microenvironments with metabolic variations that predict tumor growth. Cancer Res 70: 78-88.
39. Tsuda H, Han SM, Yang Y, Tong C, Lin YQ, et al. (2008) The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell 133: 963-977.

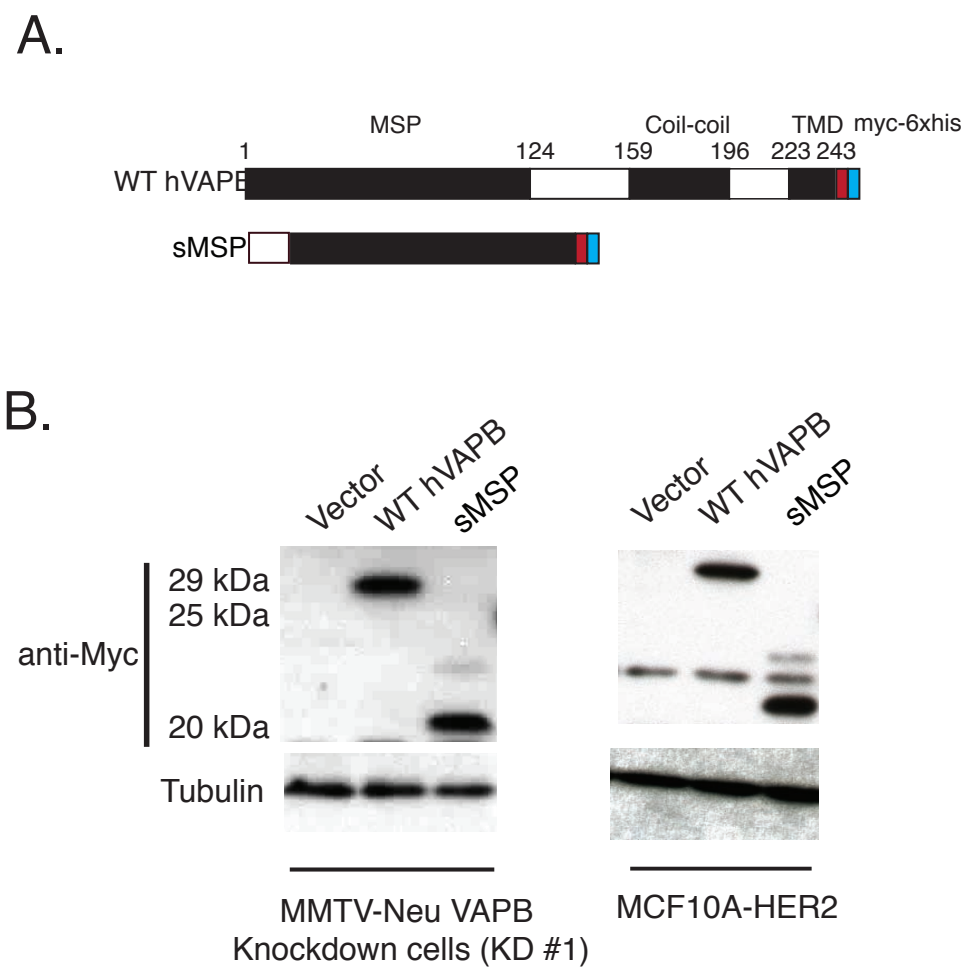


Figure 1. WT VAPB and sMSP constructs. (A) Schematic of human VAPB wildtype or sMSP cDNA tagged with Myc/ 6x His and subsequently inserted into pcLSXN_Neo retroviral vector. (B) MMTV-Neu VAPB knockdown tumor cells or MCF10A-Her2 cells were infected and selected with G418. Cell lysates were subjected to western analysis for protein expression.

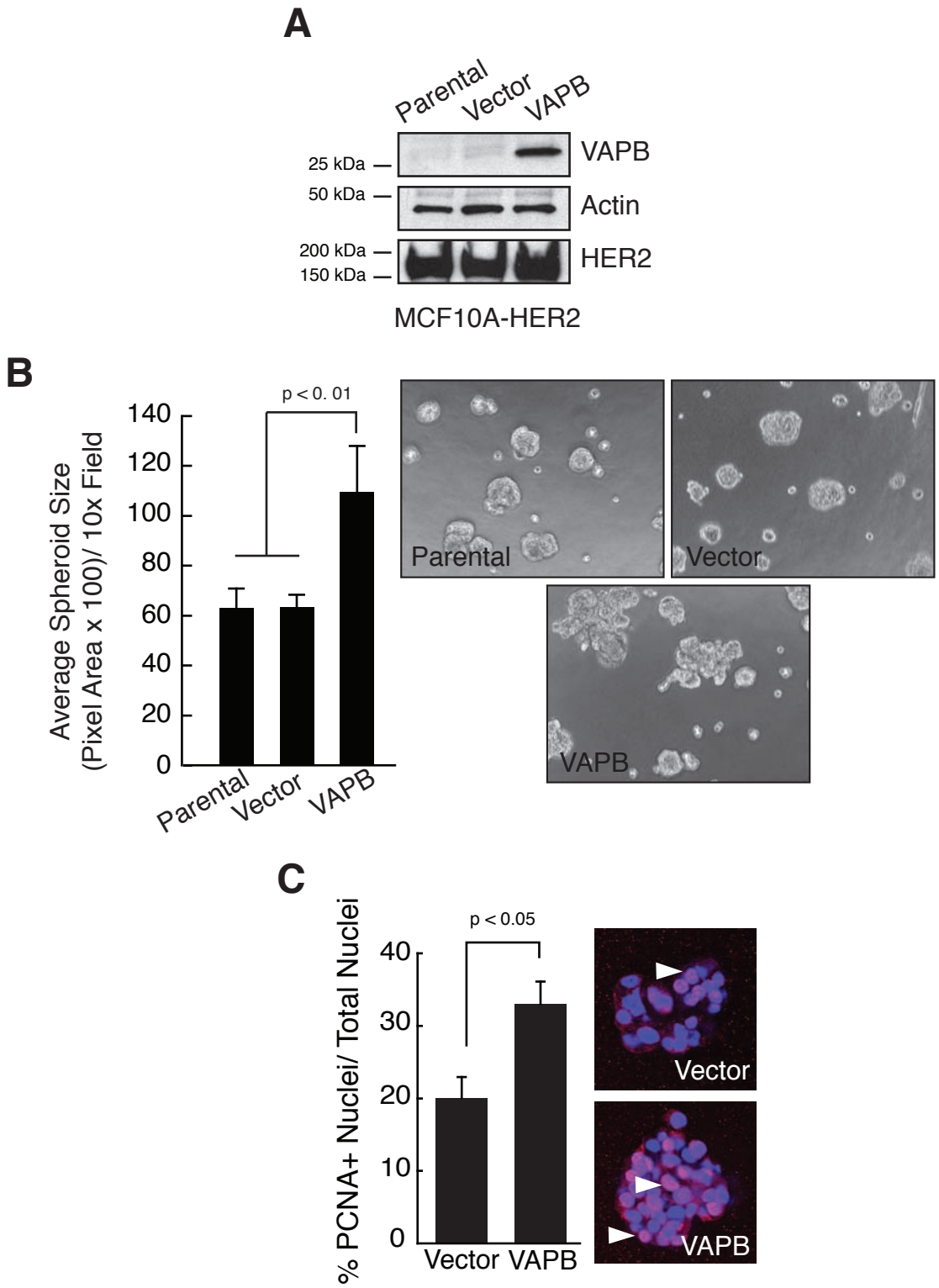


Figure 2. VAPB expression enhances spheroid size and proliferation in mammary epithelial cells. (A) VAPB was stably expressed in MCF10A-HER2 cells via retroviral transduction, as judged by western blot analysis. (B) MCF10A-HER2-VAPB spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid ($p < 0.01$, ANOVA). (C) Cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). Cell nuclei were stained blue by TO-PRO-3 nuclear stain. PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments ($p<0.05$, t test). Arrows: PCNA positive cells.

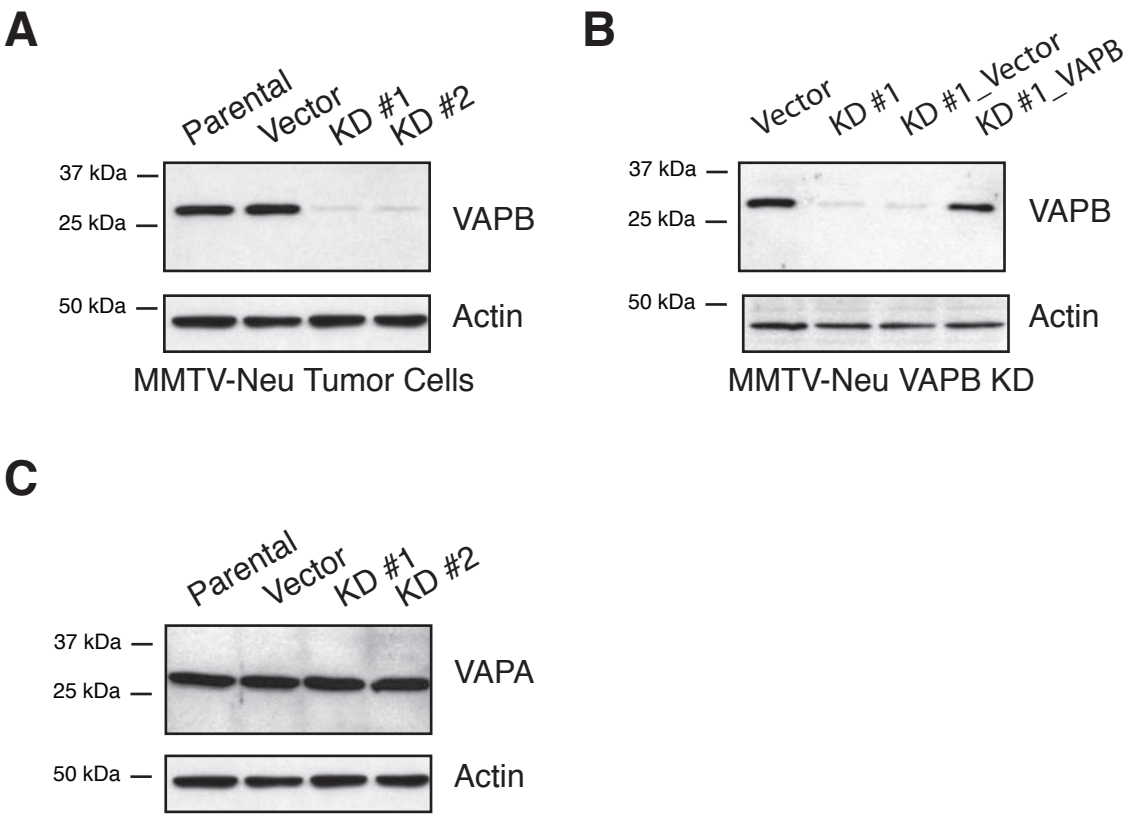


Figure 3. Specificity of VAPB shRNA knockdown. (A) VAPB expression was silenced in MMTV-Neu tumor cells by two independent lentiviral-mediated shRNAs, as judged by western blot analysis. (B) VAPB knockdown (KD #1) cells were rescued by re-expressing a full-length human VAPB cDNA via retroviral transduction. (C) VAPA protein expression was not affected by VAPB shRNA, as judged by western blot analysis.

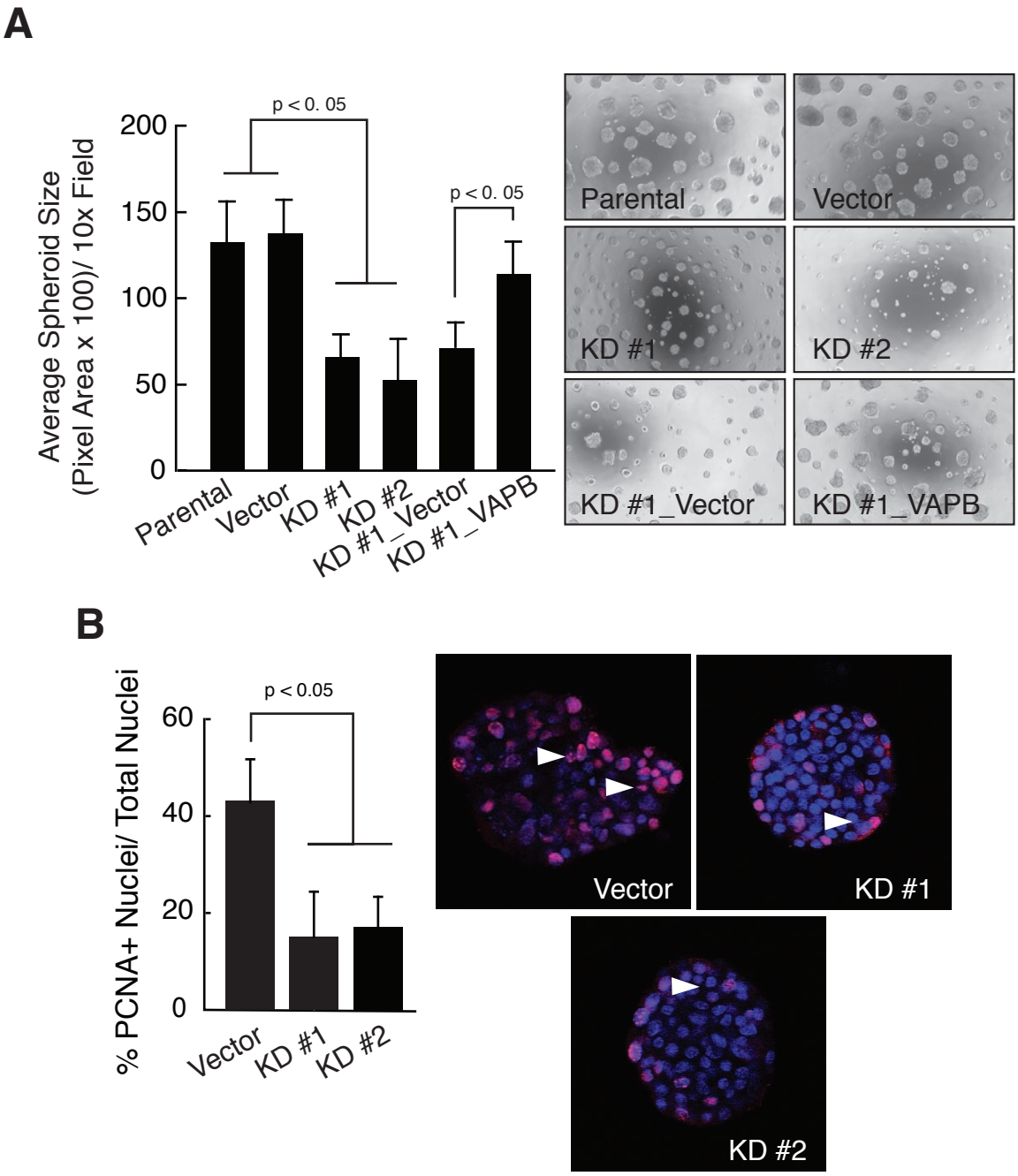


Figure 4. VAPB knockdown impairs mammary tumor spheroid growth and cell proliferation. (A) MMTV-Neu spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid ($p < 0.01$, ANOVA). (B) MMTV-Neu cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments ($p<0.05$, t test). Arrows: PCNA positive cells.

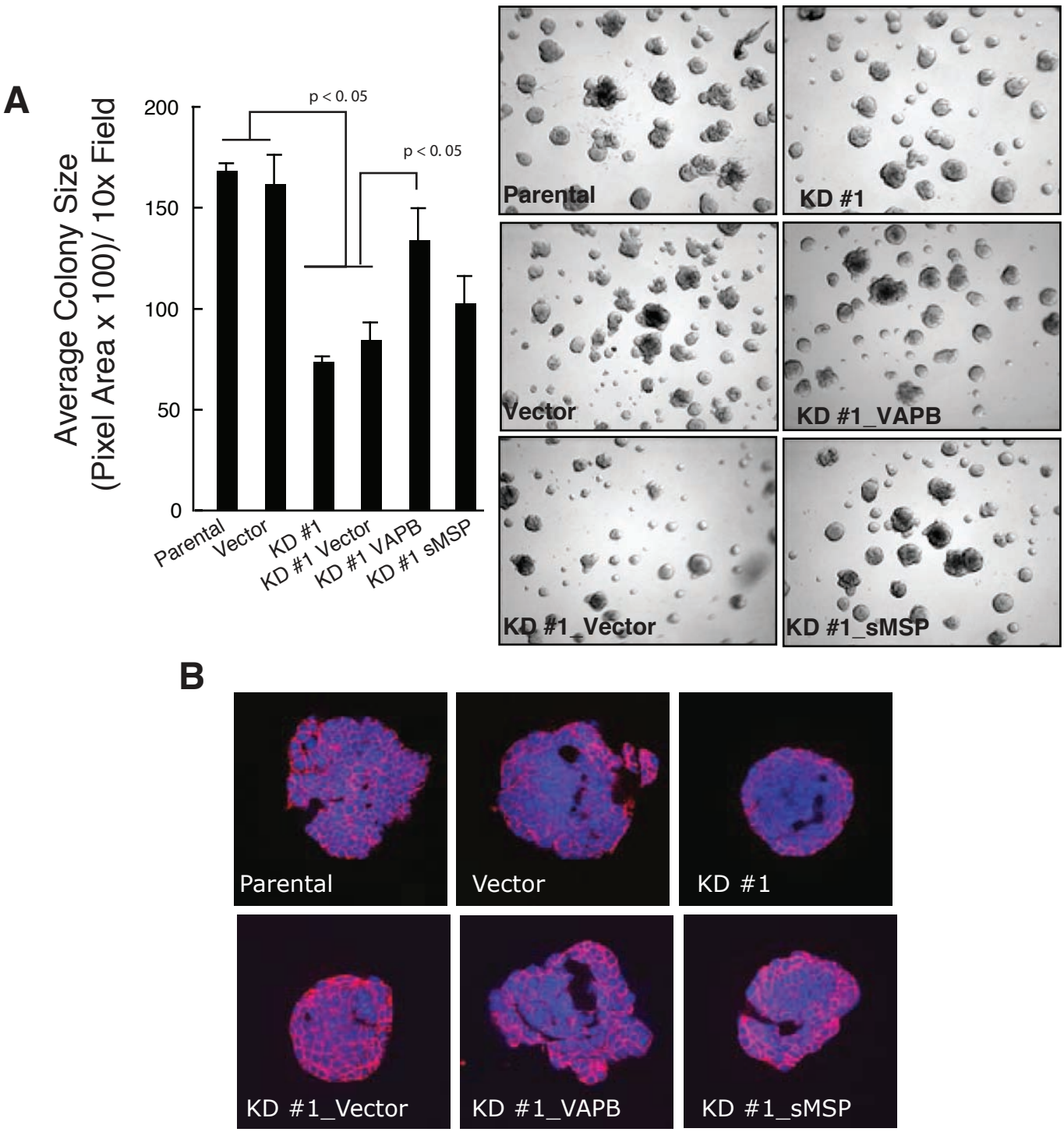


Figure 5. Partial rescue of mammosphere size in VAPB knockdown cells with VAPB or sMSP. (A) MMTV-Neu cells were retrovirally infected with WT VAPB or sMSP. We observed decreases in coloney size of VAPB mutants in 3D Matrigel culture when compared with WT VAPB ($p < 0.05$ Single Factor ANOVA). (B) Confocal analysis of colonies; E-cadherin (red) and nuclei (blue).

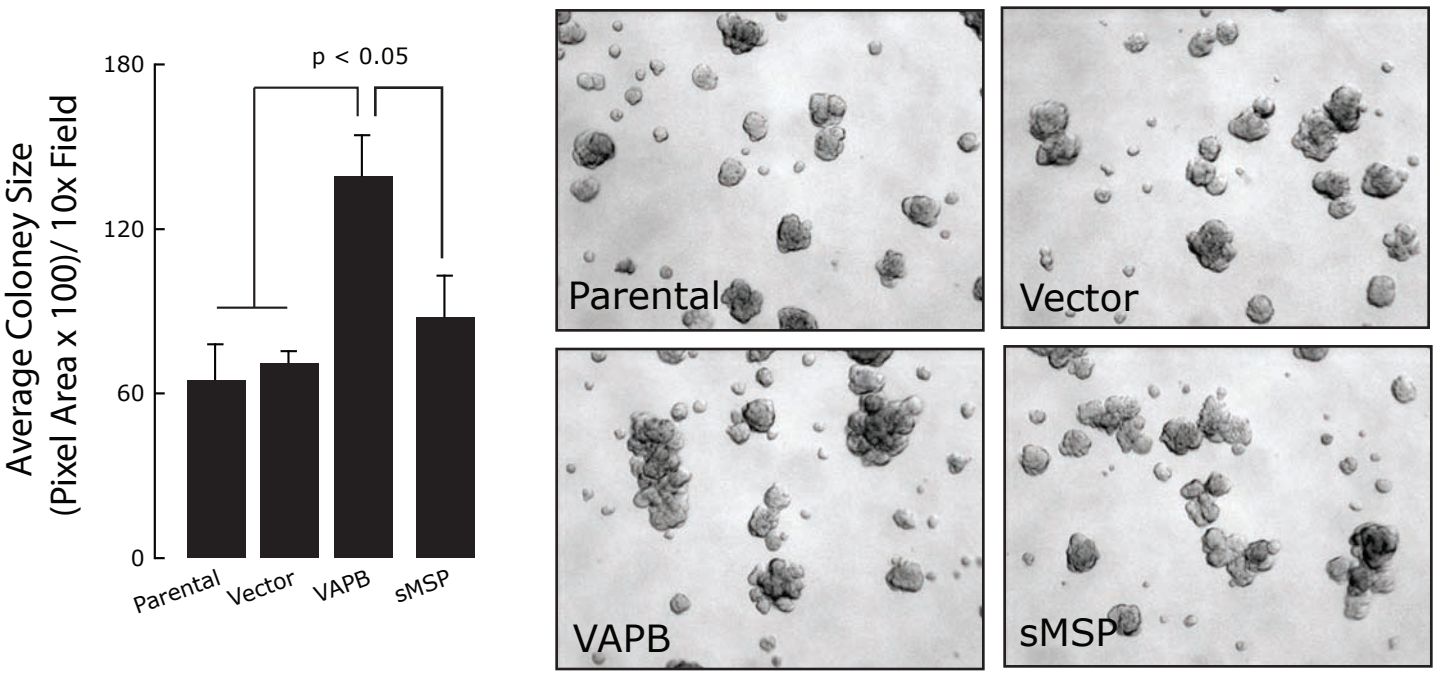


Figure 6. sMSP is not sufficient to increase spheroid size. MCF10-HER2 cells were retrovirally overexpressed with WT VAPB or sMSP. The mammosphere sizes of mutants cells were similar to parental and vector controls but were significantly decreased than WT VAPB ($p < 0.05$ Single Factor ANOVA).

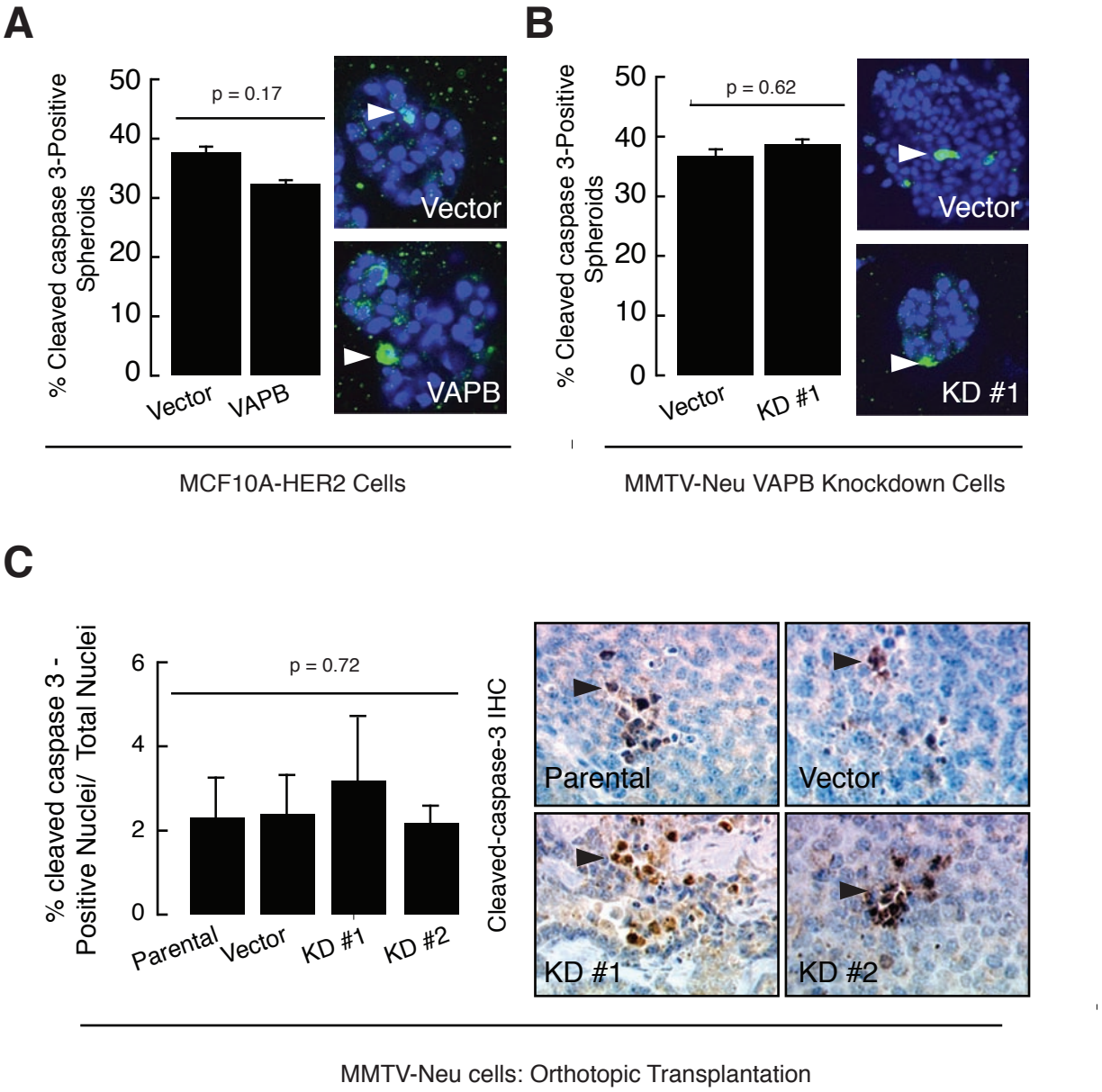


Figure 7. VAPB expression does not affect apoptosis. (A) MCF10A-HER2 or (B) MMTV-Neu knockdown cells were stained for cleaved caspase-3 at day 8 in 3-dimensional culture. Cleaved caspase-3 positive spheroids (green) were quantified by confocal microscopy analysis. No significant changes were observed in both cell lines. (C) *in vivo* analysis of apoptosis was measured by cleaved caspase-3 staining of tumor sections. Cleaved caspase -3 positive nuclei were quantified. VAPB deficiency does not significantly affect apoptosis in vivo. Arrows: cleaved caspase-3 positive cells.

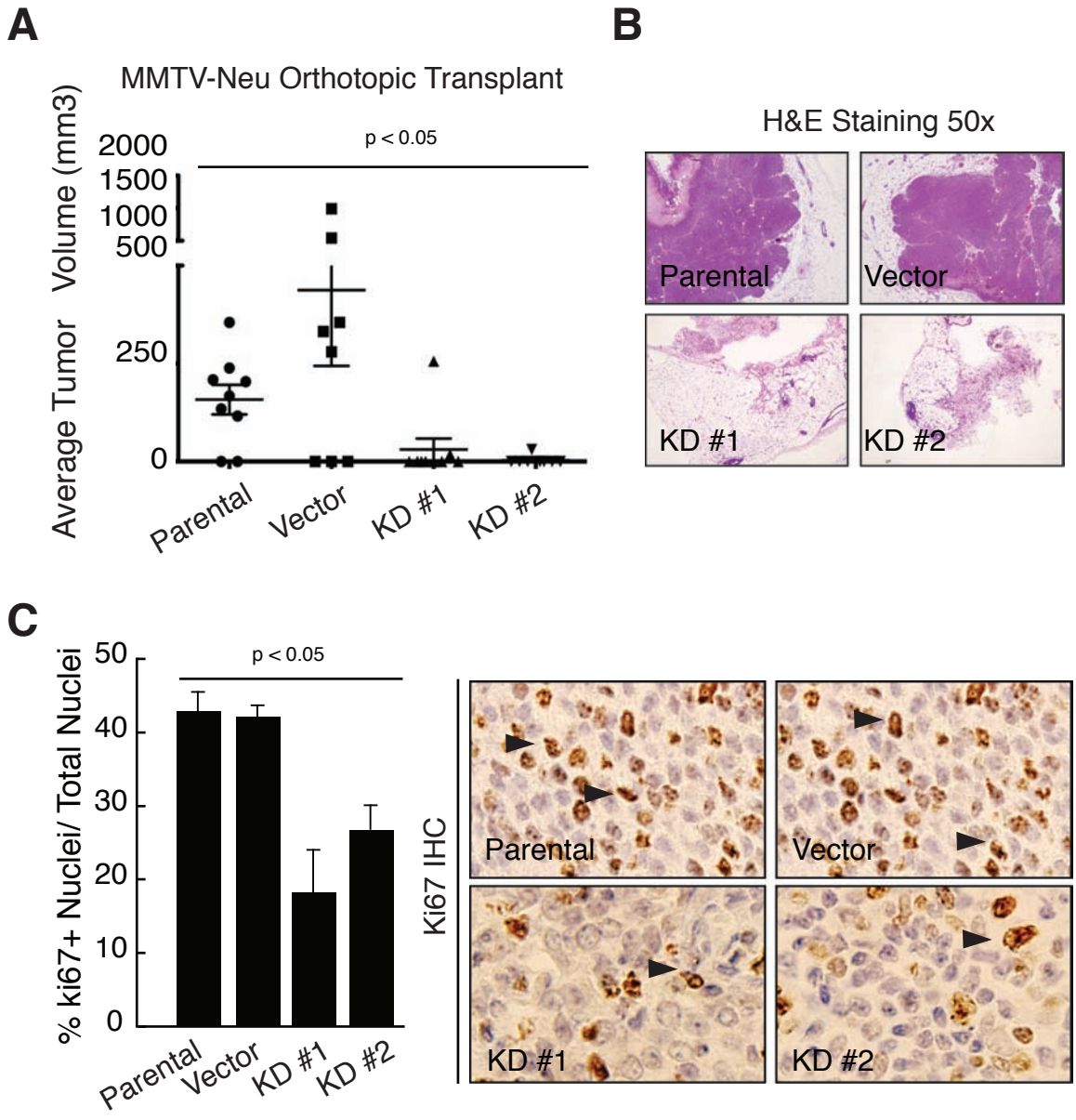


Figure 8. VAPB is required for tumor growth in vivo. (A) One million of MMTV-Neu control or VAPB knockdown cells were injected into cleared mammary gland fat pads of 3-week old FVB recipient female mice (n=8-10/experimental condition). Tumors were harvested 5 weeks after transplantation. Tumor size was measured by a caliper and tumor volumes were calculated. ($p < 0.05$, ANOVA). (B) Tumors were processed, sectioned and stained with H&E (B) or Ki67, a proliferation marker (C). Proliferating tumor cells were quantified by enumeration of Ki67+ nuclei and presented as a percentage of Ki67+ nuclei/total nuclei. VAPB knockdown cells showed a significant decrease in proliferation ($p < 0.05$, ANOVA). Arrows: Ki67 positive cells.

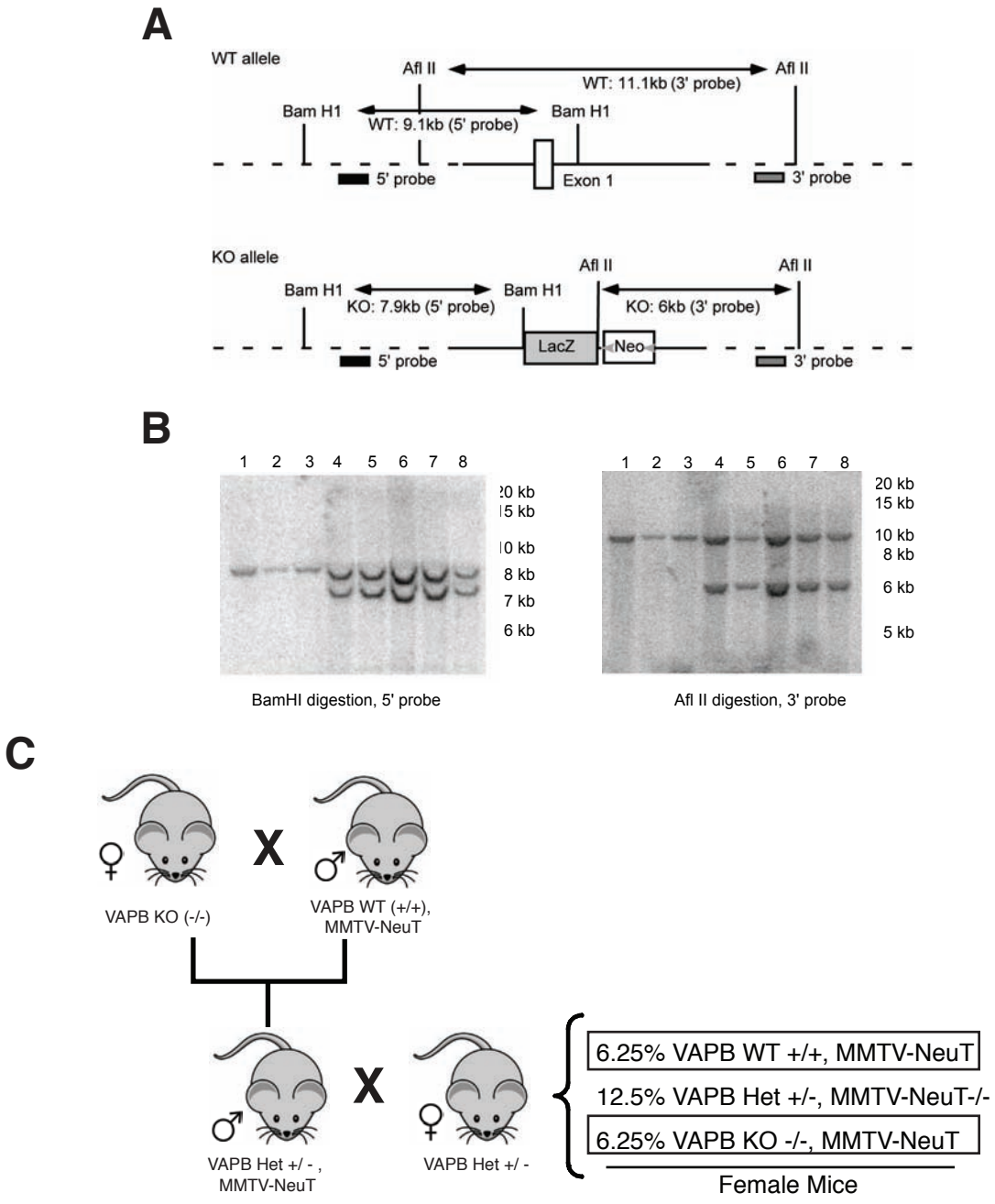


Figure 9. Generation of VAPB knockout/ LacZ knockin mice. (A) Targeting strategy. (B) Southern blotting analysis to reconfirm positive clones first identified by PCR in ES cells. Lanes 1-3 is DNA from C57Bl(6), 129/ SzEv, and B6 X 129 hybrid, respectively. (C) Breeding strategy to generate VAPB WT or VAPB KO mice that are positive for the NeuT transgene.

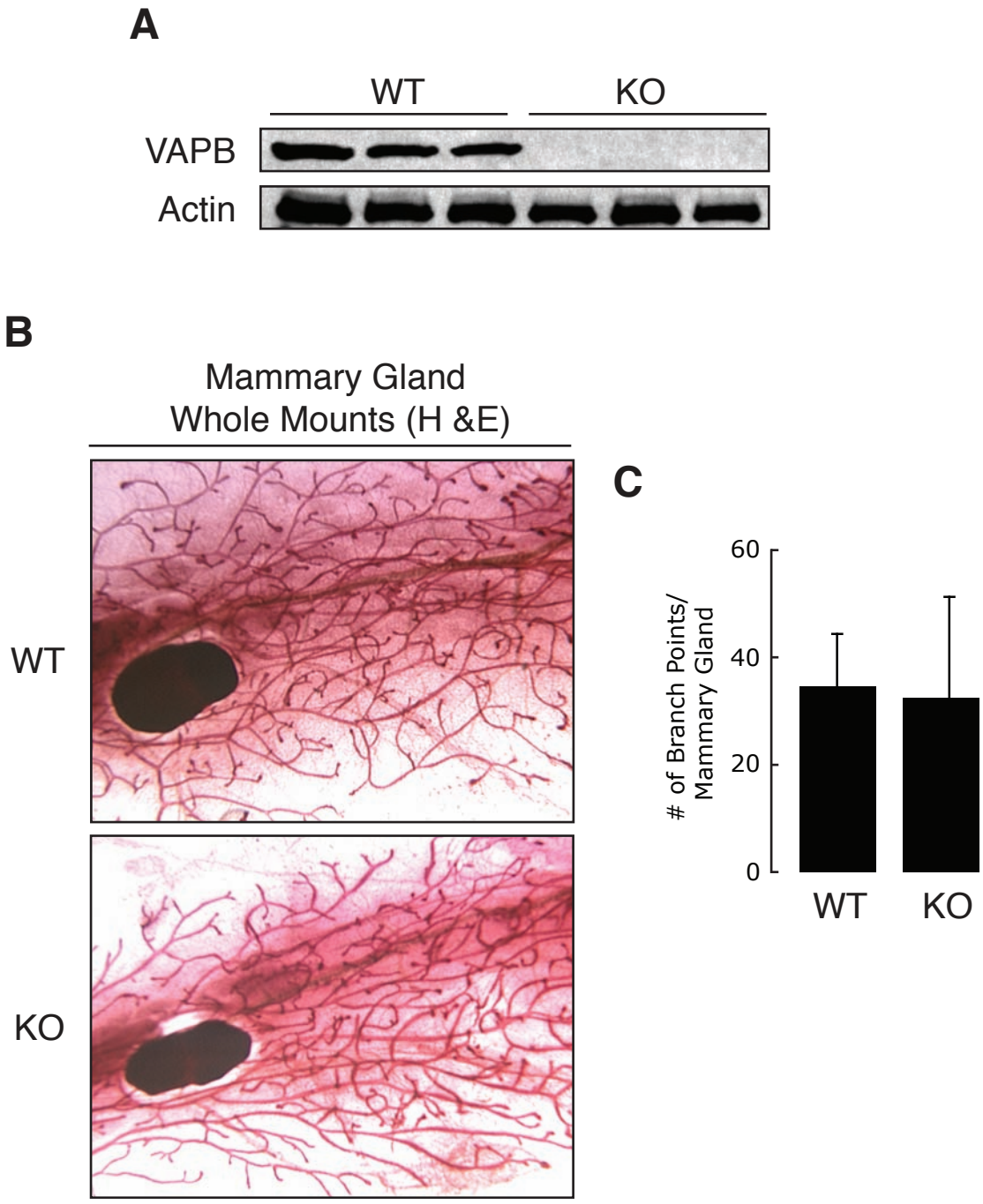


Figure 10. Normal mammary gland morphology in VAPB deficient mice. (A) VAPB protein expression from mammary gland lysates was undetectable in KO mice when compared to WT controls. (B) Whole-mount hematoxylin staining of number 4 inguinal mammary glands collected from VAPB WT and KO VAPB female animals at 8 weeks after birth and was quantified for number of branch points (C).

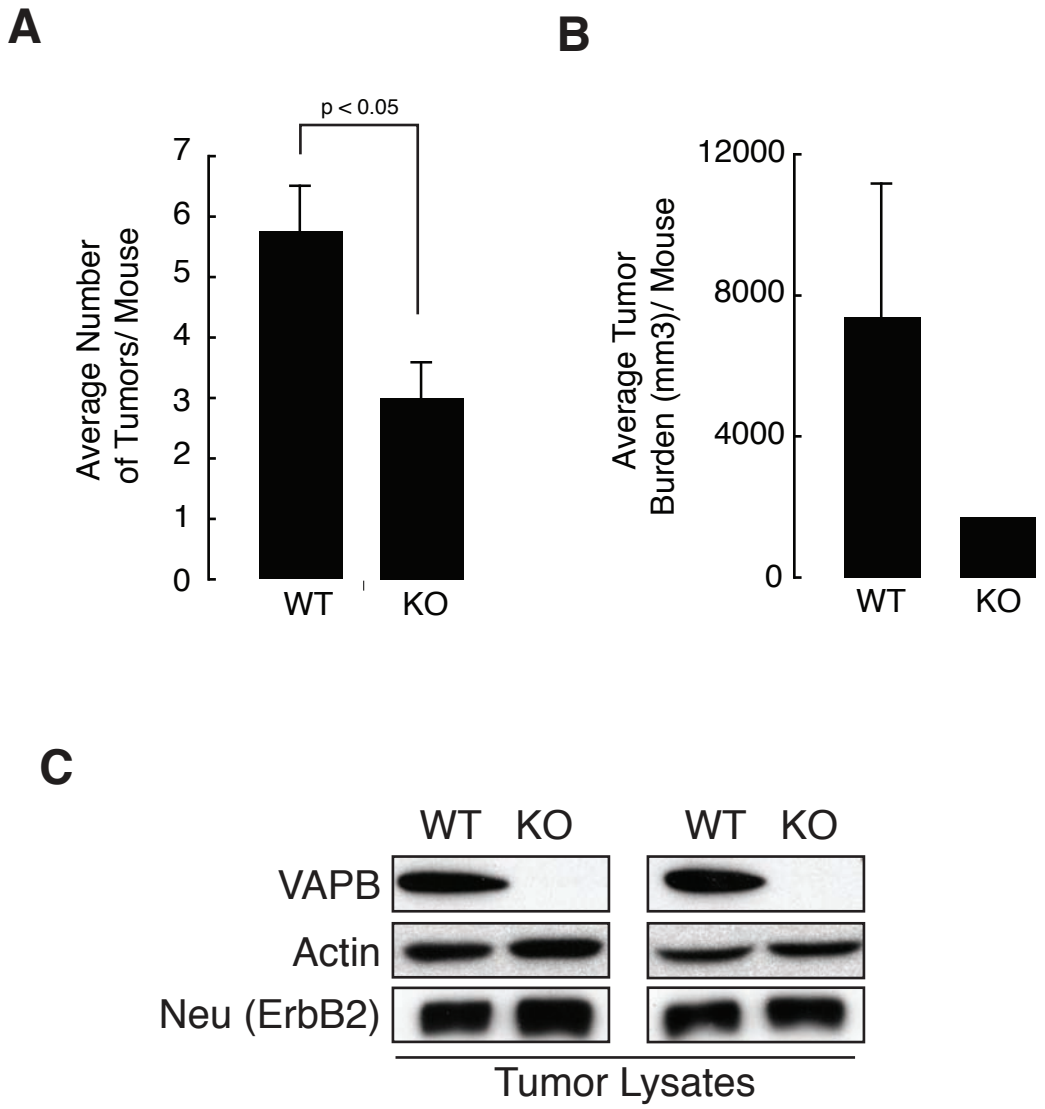


Figure 11. VAPB deficiency results in reduced tumor number and burden. (A) MMTV-NeuT/ VAPB WT or KO mice were sacrificed at 5 months of age. Tumors were harvested, enumerated and scored for size (n=4). (A) Average number of tumors per mouse was significantly reduced with VAPB deficiency ($p < 0.05$ unpaired t-test). (B) Reduced tumor burden per mouse was observed in MMTV-NeuT/ VAPB KO mice. (C) Confirmation of VAPB protein in tumor lysates.

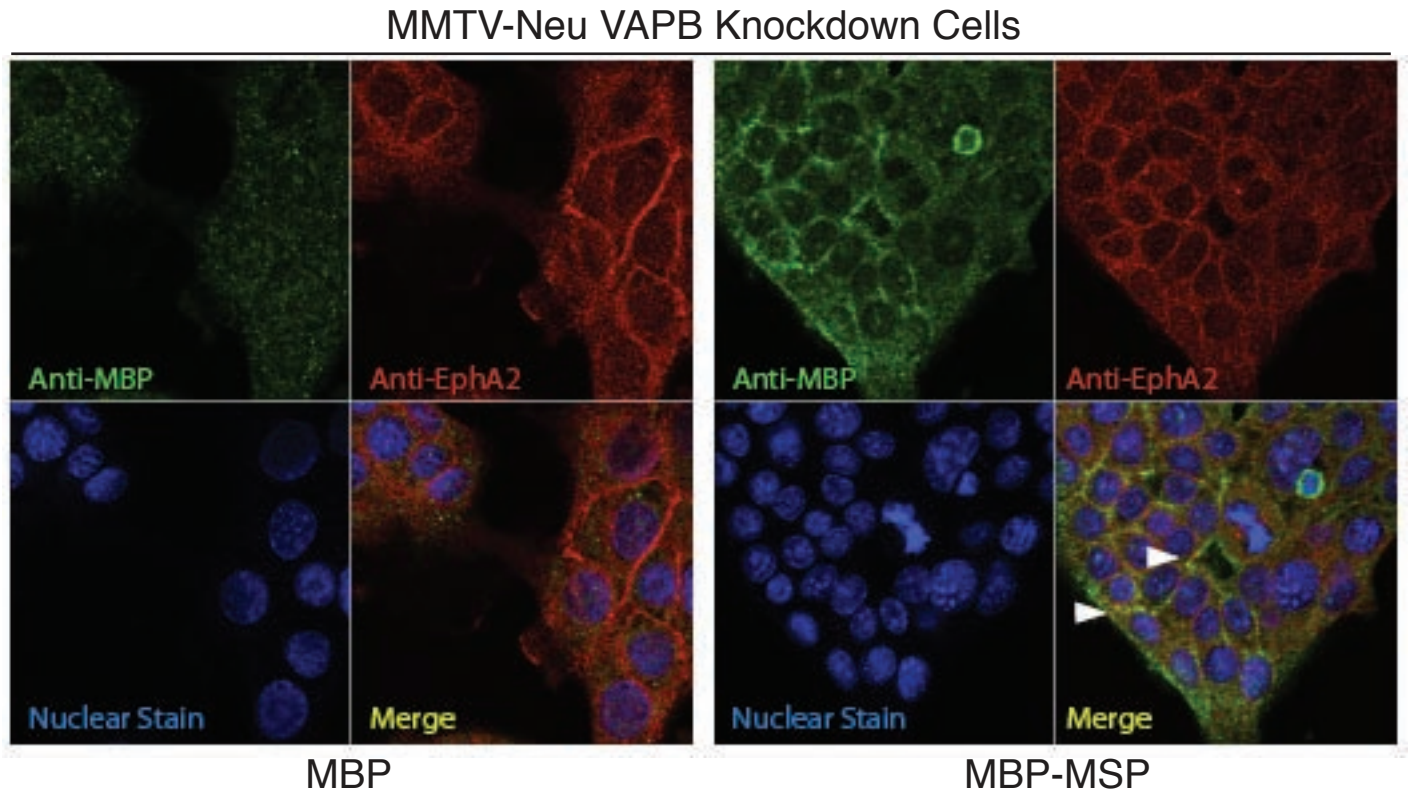


Figure 12. MBP-MSP co-localizes with EphA2. MMTV-Neu VAPB knockdown cells were incubated with 20µg of MBP control or MBP-MSP purified protein under non-permeabilizing conditions. Cells were co-stained with anti-EphA2 and anti-MBP and were analyzed by confocal microscopy. White arrows indicated co-localizaion of MBP-MSP and EphA2.

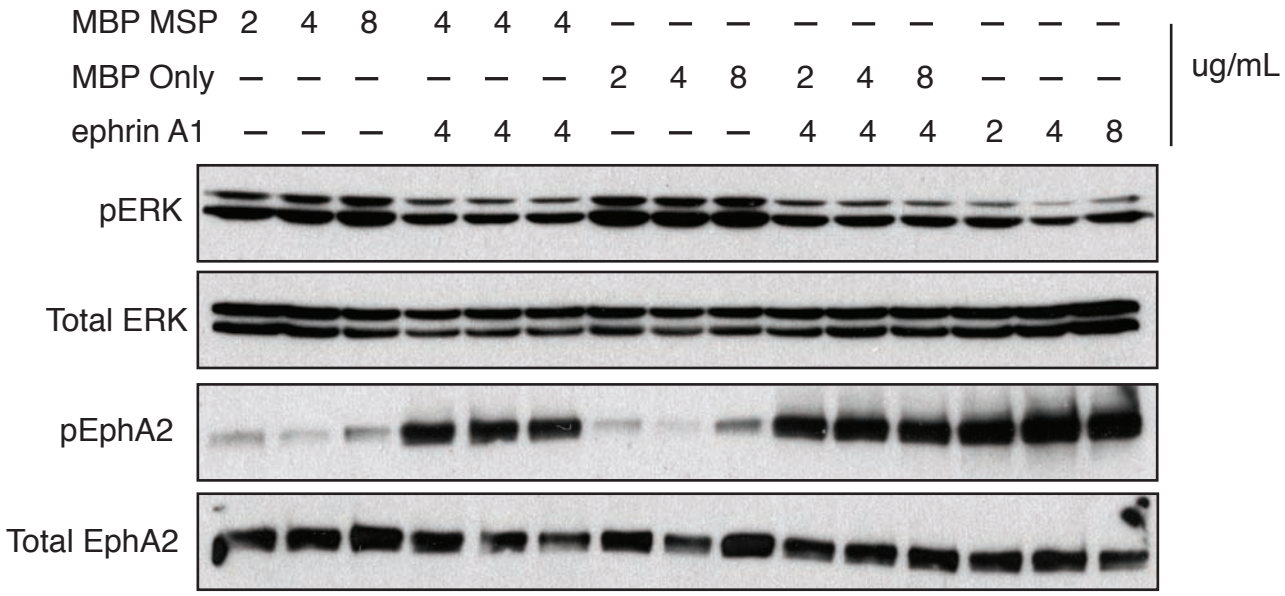


Figure 13. EphrinA1 VAPB-MSP competition assays. MMTV-Neu cells lines were stimulated with ephrinA1 in the presence of increasing amoutns of purifed MSP or control. Cell lysates were analyzed for pERK and pEphA2.

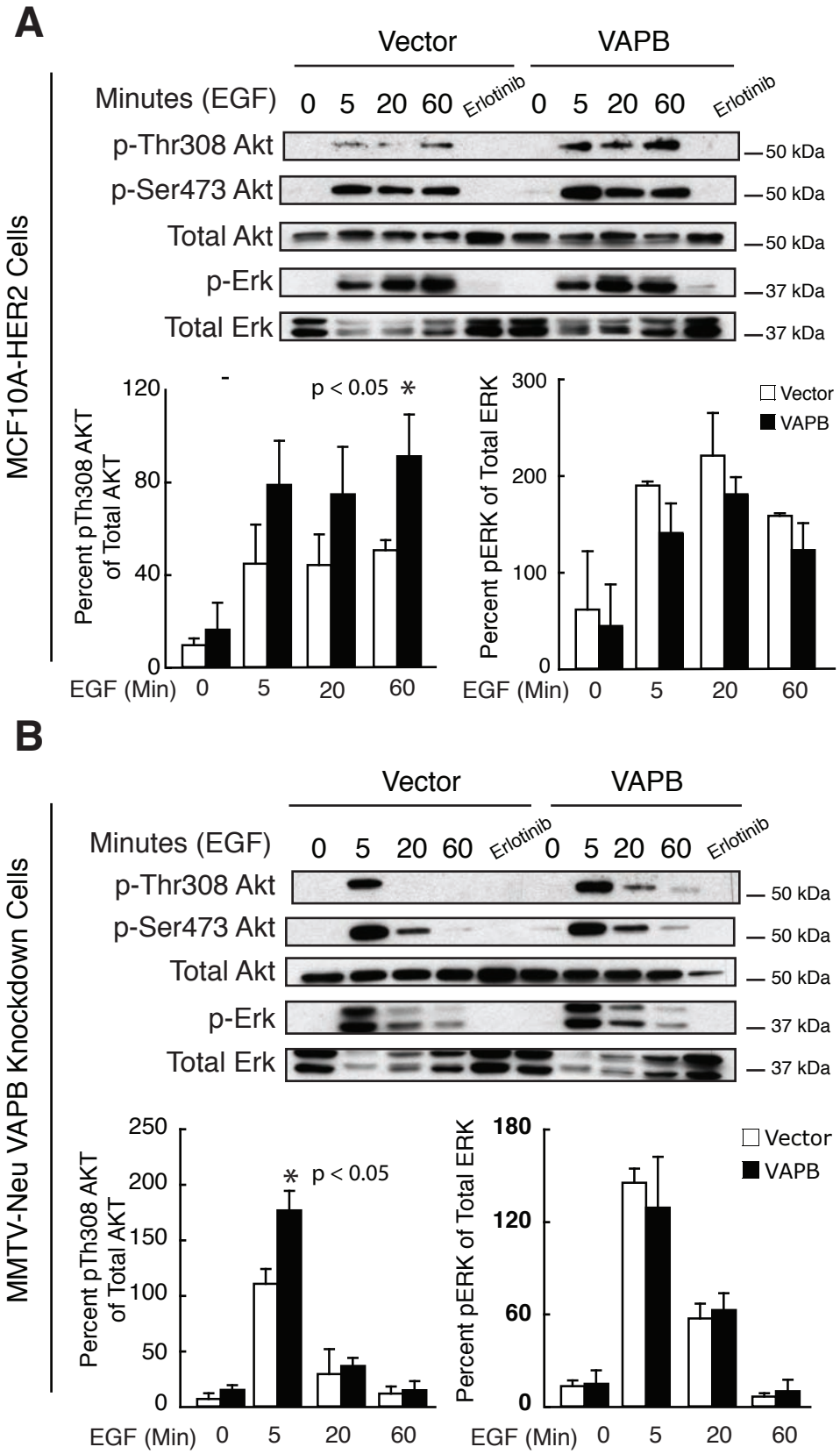


Figure 14. Analysis of AKT and ERK activities in VAPB expressing cells. (A) MCF10A cells expressing VAPB or carrying control vector were serum starved and stimulated with 20ng/mL EGF at the indicated time points. As a negative control for EGF stimulation, cells were treated for 60 minutes with EGFR inhibitor, erlotinib (1μM). Phospho-AKT and phospho-ERK levels in MCF10A-HER2 cells were measured by western blot analysis and quantified. Representative blots from 3 independent experiments are shown. (B) VAPB was knocked down in MMTV-Neu cells and re-expressed via retroviral transduction. Cells were stimulated as in (A) and phospho-AKT and phospho-ERK levels were measured by western blot analysis and quantified. Representative blots from 3 independent experiments are shown.

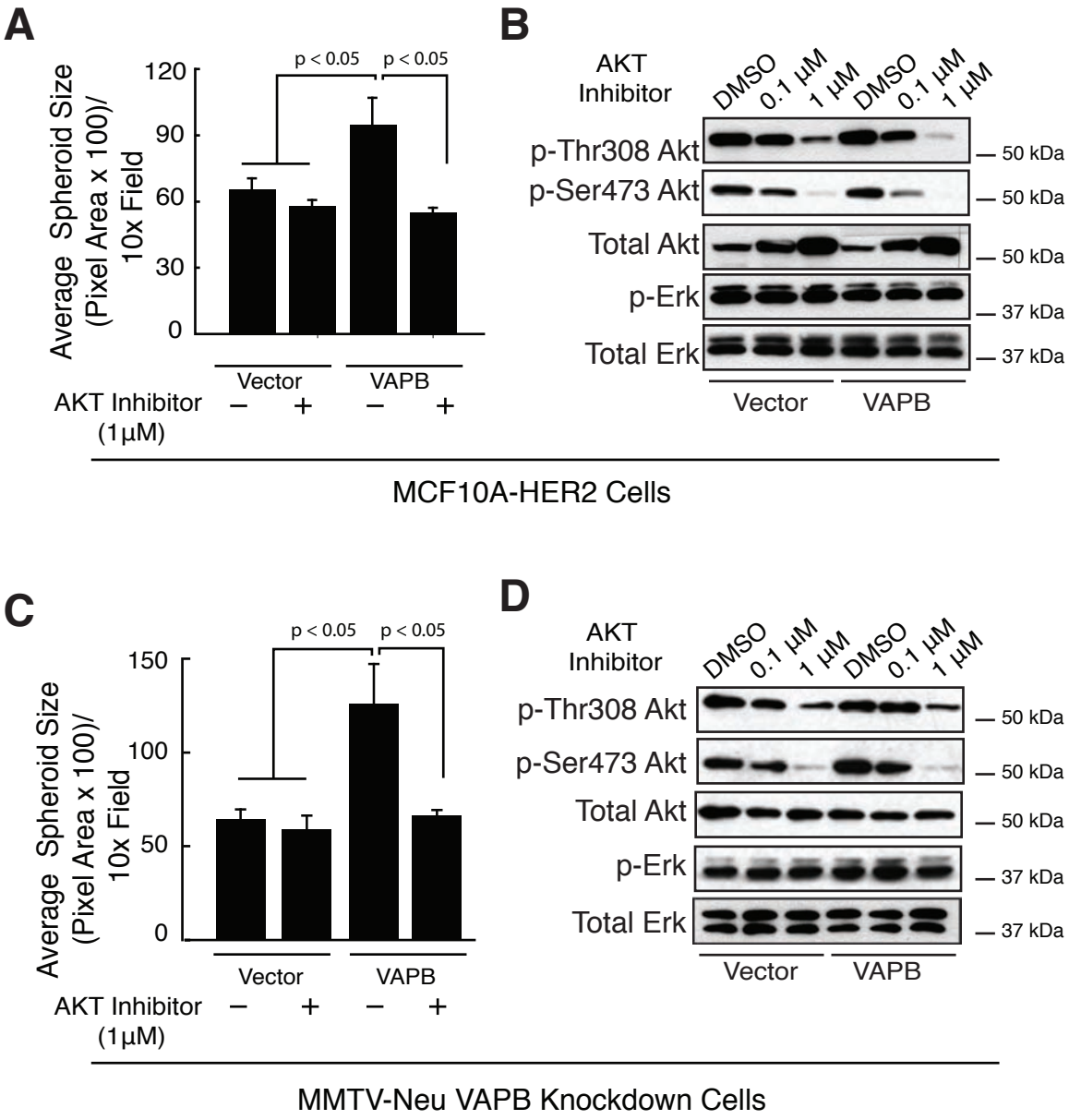


Figure 15. VAPB-dependent cell growth is mediated through AKT activity. Pharmacologic AKT inhibition significantly impaired VAPB-mediated spheroid growth in (A) MCF10A-HER2 cells and (C) MMTV-Neu VAPB knockdown cells rescued with VAPB re-expression ($p < 0.05$). Inhibition of AKT activity was confirmed by western blot analysis for phospho-AKT in (B) MCF10A-HER2 cells and (D) MMTV-Neu knockdown cells.

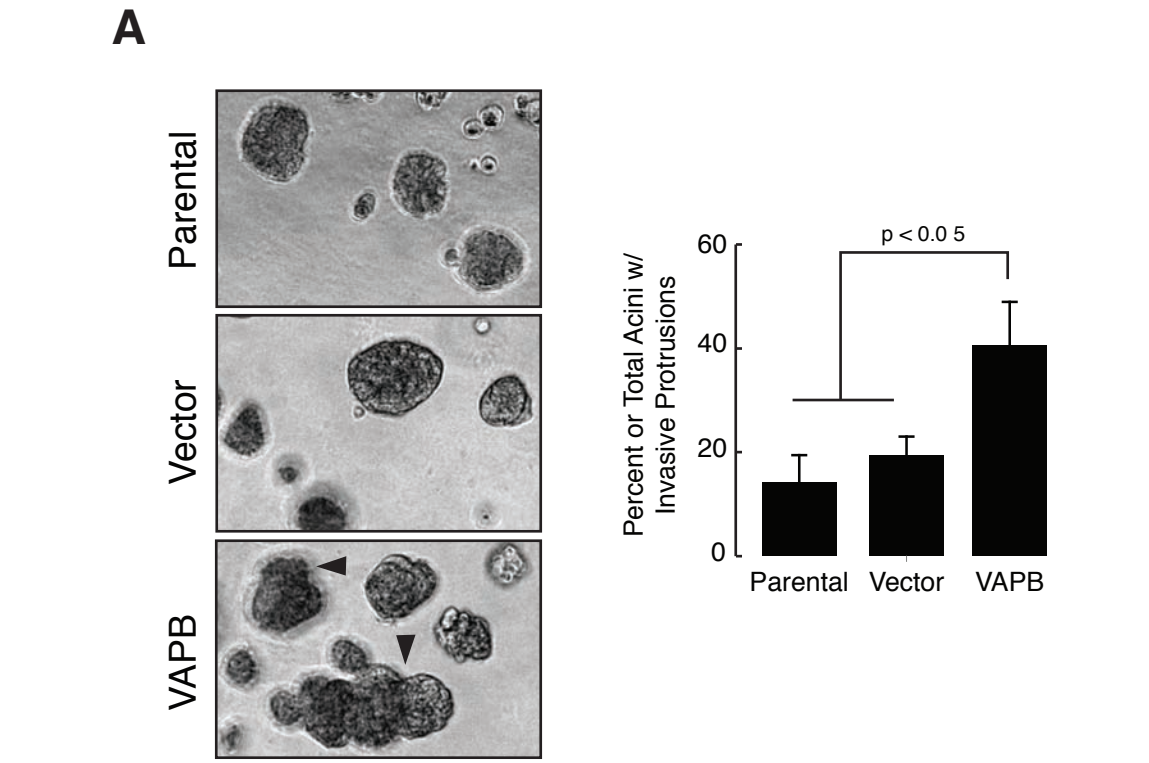


Figure 16. VAPB induces invasive-like growth into the extracellular matrix. (A) MCF10A-HER2-VAPB cells were cultured in Matrigel. At day 9, cultures were photodocumented and scored for invasive growth (black arrows) as previously described (Chatterjee et al, 2012). Percentage of acini displaying invasive protrusions were quantified from three independent experiments ($p < 0.05$, t-test) (B) Acini were stained at day 9 for TO-PRO-3 nuclear stain (blue), E-cadherin (red) and laminin Vγ2 (green) and imaged by confocal microscopy. Confocal analysis revealed MCF10A-HER2 VAPB acini displayed normal E-cadherin localization at the cell junction, but diminished laminin V localization at areas of invasive growth (white arrow).

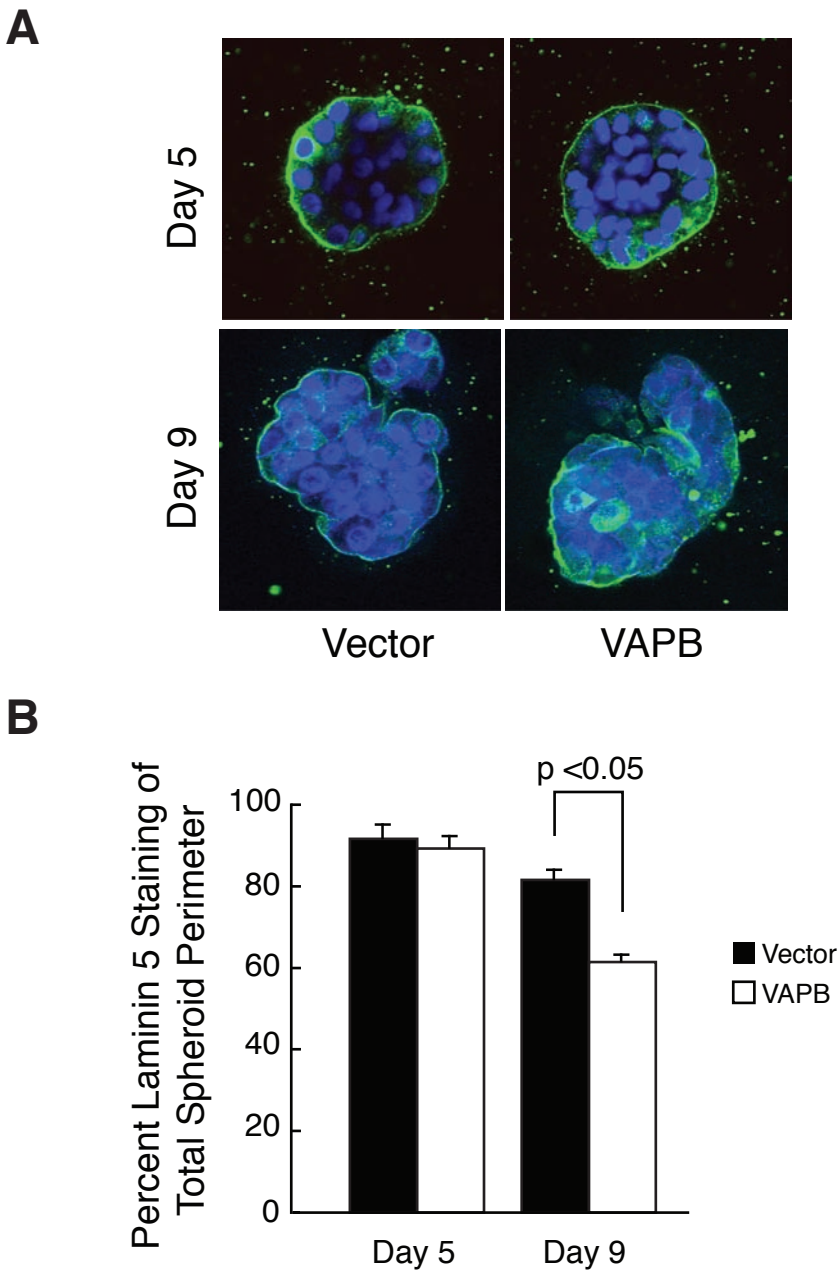


Figure 17. VAPB expression disrupts basement membrane integrity. (A) MCF10A-HER2 cells were cultured in Matrigel and stained at day five and day nine for lamininVγ2 (green) and TO-PRO-3 nuclear stain (blue) and analyzed by confocal microscopy. At day five both vector and VAPB expressing cells displayed continuous laminin V staining around each spheroid which was significantly reduced by day nine in VAPB-expressing cells only. (B) Quantification of 30 randomly selected spheroids from two independent experiments is shown in the lower panel ($p < 0.05$ ANOVA).

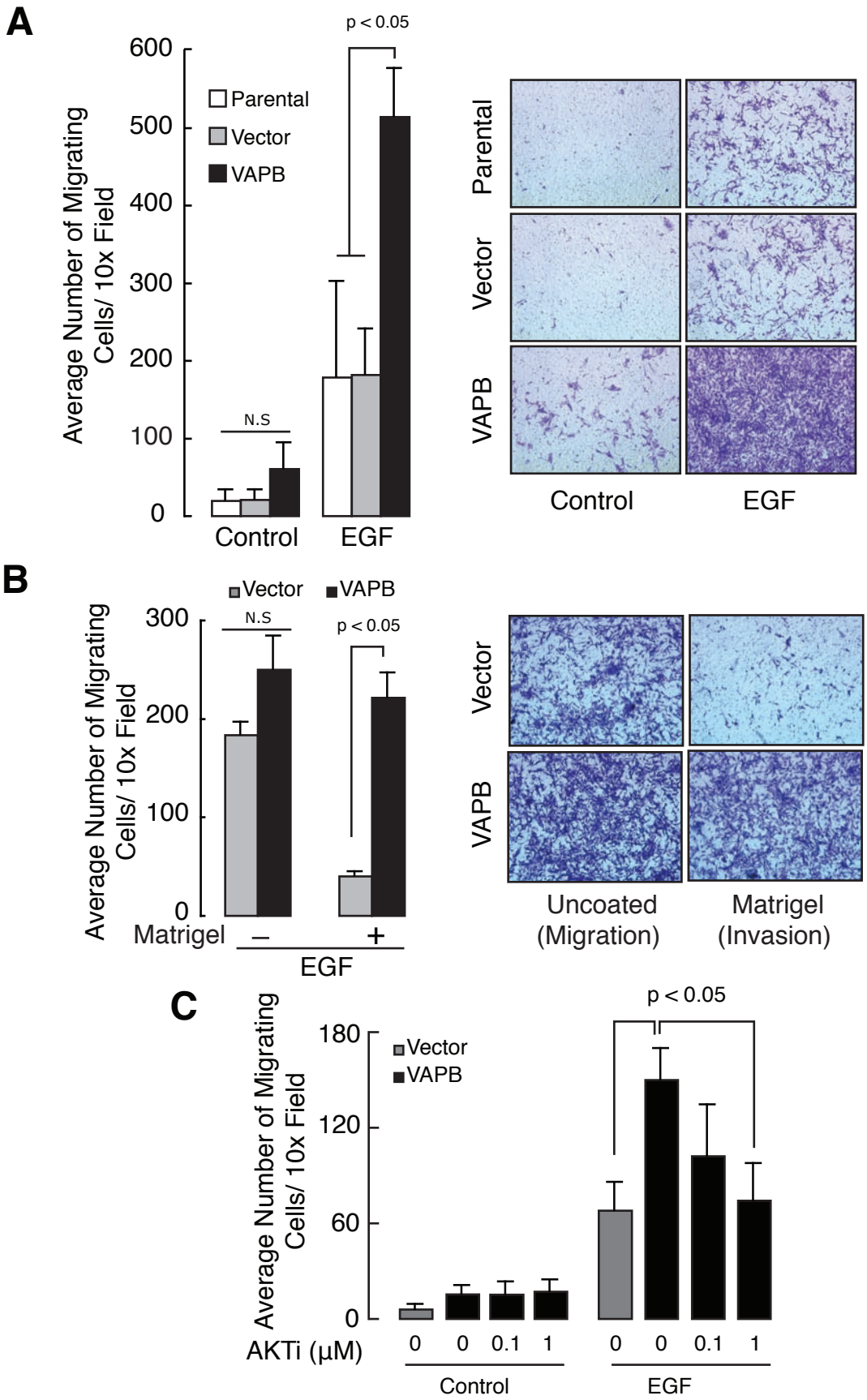


Figure 18. VAPB elevates the invasive potential of MCF10A-HER2 cells. (A) VAPB significantly increased EGF-induced (20ng/mL) invasion through Matrigel-coated, Boyden chambers (Transwells). (B) EGF- induced migration was assayed using uncoated Transwell filters. No significant difference was observed between vector and VAPB-expressing cells. (C) Pharmacologic inhibition of AKT significantly impaired EGF-induced invasion in VAPB expressing cells. Inhibition of AKT activity was confirmed by western blot analysis as shown in Figure 2.7 B. All results were quantified from three independent experiments ($p < 0.05$ ANOVA).

OPEN ACCESS Freely available online

PLOS ONE

VAMP-Associated Protein B (VAPB) Promotes Breast Tumor Growth by Modulation of Akt Activity

Meghana Rao[1], Wenqiang Song[2], Aixiang Jiang[4,5], Yu Shyr[4,5], Sima Lev[7], David Greenstein[8], Dana Brantley-Sieders[2], Jin Chen[1,2,3,5,6]*

1 Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **2** Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **3** Department of Cellular and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **4** Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **5** Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **6** Veterans Affairs Medical Center, Tennessee Valley Healthcare System, Nashville, Tennessee, United States of America, **7** Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, **8** Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota, United States of America

Abstract

VAPB (VAMP- associated protein B) is an ER protein that regulates multiple biological functions. Although aberrant expression of VAPB is associated with breast cancer, its function in tumor cells is poorly understood. In this report, we provide evidence that VAPB regulates breast tumor cell proliferation and AKT activation. VAPB protein expression is elevated in primary and metastatic tumor specimens, and VAPB mRNA expression levels correlated negatively with patient survival in two large breast tumor datasets. Overexpression of VAPB in mammary epithelial cells increased cell growth, whereas VAPB knockdown in tumor cells inhibited cell proliferation *in vitro* and suppressed tumor growth in orthotopic mammary gland allografts. The growth regulation of mammary tumor cells controlled by VAPB appears to be mediated, at least in part, by modulation of AKT activity. Overexpression of VAPB in MCF10A-HER2 cells enhances phosphorylation of AKT. In contrast, knockdown of VAPB in MMTV-Neu tumor cells inhibited pAKT levels. Pharmacological inhibition of AKT significantly reduced three-dimensional spheroid growth induced by VAPB. Collectively, the genetic, functional and mechanistic analyses suggest a role of VAPB in tumor promotion in human breast cancer.

Citation: Rao M, Song W, Jiang A, Shyr Y, Lev S, et al. (2012) VAMP-Associated Protein B (VAPB) Promotes Breast Tumor Growth by Modulation of Akt Activity. PLoS ONE 7(10): e46281. doi:10.1371/journal.pone.0046281

Editor: Waldemar Debinski, Wake Forest University, United States of America

Received June 4, 2012; **Accepted** August 28, 2012; **Published** October 1, 2012

Funding: This work was supported by the Department of Veterans Affairs (VA) through a VA Merit Award (JC), National Institutes of Health RO1 grants CA95004 & CA114301 (JC), a KO1 award CA1179151 (DBS), and a Department of Defense predoctoral fellowship W81XWH-10-1-0256 (MR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jin.chen@vanderbilt.edu

Introduction

Vesicle associated membrane protein associated protein B (VAPB) is a highly conserved type II integral membrane protein that belongs to the VAP protein family [1,2] and primarily localizes to the endoplasmic reticulum (ER) and cis-Golgi [3,4]. Studies of VAP-interacting proteins in yeast and in higher organisms implicate VAP proteins in a diverse array of cellular processes. VAP proteins function in the regulation of neurotransmitter release, vesicle trafficking, lipid binding and transfer proteins, maintainance of ER/golgi architecture and the unfolded protein response (UPR) [5,6,7,8]. Recent studies in C. elegans and Drosophila discovered that the MSP domain of VAPB can be cleaved, secreted, and act as a ligand for Eph receptor tyrosine kinases [9]. A single missense mutation within the human VAPB gene is associated in a familial form of atypical amyotrophic lateral sclerosis (ALS) [10,11,12], triggering a renewed interest in the VAPB protein and its cellular function in human pathologies.

In addition to familial ALS, VAPB expression has been observed with cancer. A genome-wide microarray analysis of 50 human breast cancer cell lines and 145 clinical specimens revealed that VAPB is often amplified or overexpressed in breast cancer [13,14]. As a potential Eph receptor ligand, the MSP domain of the VAPB protein could affect tumor growth and invasion through modulation of Eph receptor activity, which is commonly dysregulated in cancer [15,16]. Furthermore, as VAPB also functions in protein secretion and vesicle trafficking, tumor cells may rely on this pathway for receptor localization and growth factor secretion in order to sustain growth [17]. Despite strong indications of the consequences of VAPB expression in cancer, a direct role of VAPB in tumor growth has not been investigated.

In this report, we studied the role of VAPB in breast cancer. We analyzed the expression of VAPB in both a breast cancer tissue microarray and two published large mRNA expression datasets and correlated VAPB expression with clinical outcomes. To determine the causal role of VAPB in cancer, we overexpressed VAPB in mammary epithelial cells or stably knocked down VAPB in tumor cells. Cell proliferation, apoptosis, spherical growth in 3D culture, and tumor growth in vivo were analyzed. Finally, we identified molecular mechanisms by which VAPB regulates tumor cell proliferation.

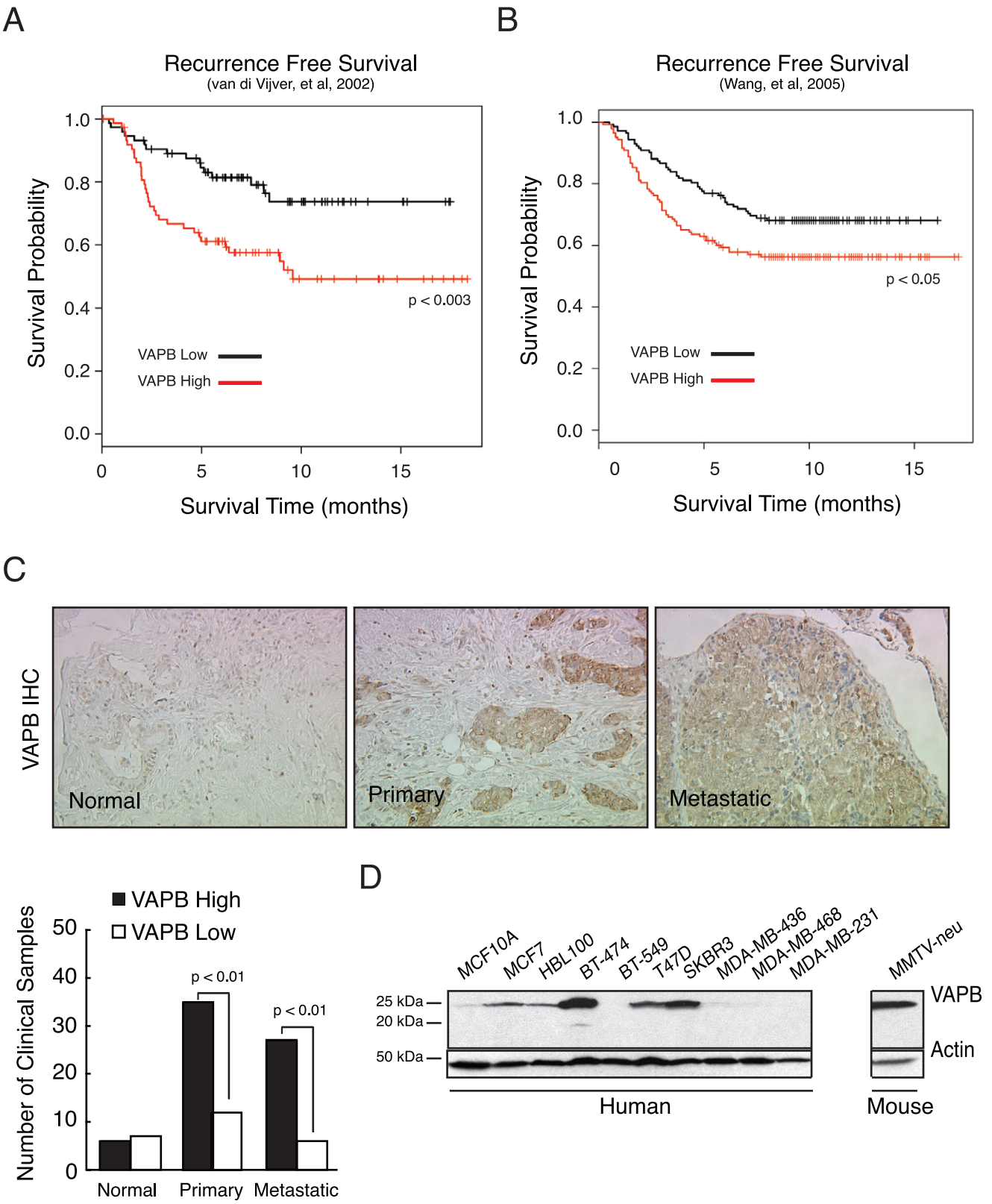


Figure 1. Analysis of VAPB expression in human breast cancer. VAPB expression levels were analyzed in two published breast cancer microarray datasets [19–20]. Correlation of VAPB expression with clinical outcome was analyzed by Kaplan-Meier survival curve using the van de Vijver dataset (A) [19] and the Wang dataset (B) [20] (C) VAPB protein expression was analyzed in human invasive ductal carcinoma samples on commercial tissue microarrays by immunohistochemistry using a previously validated anti-VAPB antibody, as described in Materials and Methods (D) Western analysis of VAPB protein in human breast cancer cell lines.
doi:10.1371/journal.pone.0046281.g001

Materials and Methods

Antibodies, Reagents and Plasmids

Antibodies against the following proteins were used: anti-VAPB (K-16), anti-VAPA (K-14), anti-actin (I-19), anti-β-tubulin (Santa Cruz Biotechnologies), and anti-PCNA (NeoMarkers), anti-Myc, anti-cleaved casepase 3, anti-pThr308AKT, anti-pSer473AKT, anti-pERK, anti-AKT, anti-ERK (Cell Signaling Technologies). Lentiviral control and VAPB shRNA plasmids KD# 1 (5′-GCACACACAAATATAGCATAA-3′) and KD# 2 (5′-CGGAAGACCTTATGGATTCAA-3′) and VAPB cDNA were obtained from OpenBiosystems. Growth factor-reduced Matrigel and TO-PRO3 was purchased from BD Biosciences and Invitrogen, respectively. The AKT 1/2 inhibitor 5J8/0360263-1 was produced by the Vanderbilt University Department of Chemistry as described previously [18].

Human mRNA Expression Profiling and Tissue Microarray Protein Analyses

Analysis of VAPB expression in human breast cancer datasets [19,20] was performed in collaboration with the Vanderbilt-Ingram Cancer Center's Biostatistics Core Resource. VAPB "high" or "low" expression was defined as top or bottom quartiles of tumors expressing VAPB. Expression levels were analyzed in relation to overall and/or recurrence-free survival using Log Rank and Cox analyses. Statistical analyses were performed using Software: R2.12.1. The survival curves from Kaplan-Meier was created and plotted by the function "survfit" under R package "survival." P values shown on KM plots were calculated based on log rank test between two survival curves of high or low expression groups.

Breast tumor spectrum Tissue Microarrays (TMAs; BR480) were purchased from US Biomax, Inc. (Rockville, MD). Immunohistochemical staining for VAPB was performed as described previously [15,21] using a previously validated rabbit anti-VAPB antibody [22].Briefly, tumor sections were re-hydrated and subjected to thermal antigen retrieval in citrate buffer (2 mM citric acid, 10 mM sodium citrate, pH 6.0) using a PickCell Laboratories 2100 Retriever as per manufacturer's instructions. Endogenous peroxidases were quenched by incubation in 3% H_2O_2 solution for 30 minutes. After blocking, sections were incubated with anti-VAPB (1:25, produced in the Lev lab) overnight at $\mathring{4}$C, followed by a biotinylated goat anti-rabbit secondary antibody (1:200, BD Pharmingen, San Diego, CA), an avidin-peroxidase reagent (Life Technologies/Molecular Probes, Carlsbad, CA), and stained with liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Life Technologies/Zymed Laboratories). Relative expression in TMAs was scored using a continuous scale as [22] follows: 0 = 0−10% positive tumor epithelium, 1 = 10−25% positive tumor epithelium, 2 = 25−50% positive tumor epithelium, and 3 = >50% positive tumor epithelium/core. Differential expression between tissue samples was quantified and analyzed statistically (Chi square analysis).

Cell Culture and Generation of Stable VAPB Knockdown or Overexpression Cell Populations

MMTV-Neu tumor cells were isolated as previously described [23] and grown in DMEM/F-12 (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone), 5 ng/mL estrogen (Sigma-Aldrich), 5 ng/mL progesterone (Sigma-Aldrich), 5 ng/mL EGF (PreproTech), 5 μg/mL insulin (Sigma-Aldrich) with penicillin/streptomycin. For generation of stable VAPB knockdown cells, Vector-only (pLKO.1) or VAPB shRNA lentivirus was produced by co-transfecting HEK-293T cells with pLKO.1 or pLKO.1-shRNAs, along with packaging plasmids psPAX2 and pMD.2G, using Lipofectamine. Viral supernatants were collected 48 and 72 hours post transfection and used to infect MMTV-Neu tumor cells. MMTV-Neu cells were selected for plasmid inclusion by culturing cells in growth media containing 5 μg/mL of puromycin (Sigma-Aldrich). For re-expression of VAPB, VAPB cDNA (open reading frame) was subcloned into pCLSXN retroviral vector that contains a Neomycin resistance gene, allowing for G418 (300 μg/mL for 6 days) selection in puromycin-resistant populations. Viral supernatant was collected as described above from HEK 293T cells that were co-transfected with pCLSXN-Vector (10 μg) only or pCLSXN-VAPB (10 μg) and pCLAmpho plasmids (10 μg) using Lipofectamine 2000.

MCF10A cells were obtained from American Type Culture Collection (ATCC) and cultured as previously described [24]. MCF10A-HER2 cells were generated via retroviral transduction of the HER2 proto-oncogene [25]. Overexpression of VAPB in MCF10A-HER2 cells was achieved by infecting cells with pCLXSN-Vector or -VAPB retrovirus and selected in media containing 300 μg/mL G418 for 6 days.

Analysis of 3-dimensional Spheroid Culture

MMTV-Neu tumor cells or MCF10A-HER2 cells were plated on a solidified layer of Matrigel (2 mm thick) using an 8-well, glass chamber slide (Thermo Scientific) as previously described [15,24]. Fresh MMTV-Neu growth media or MCF10A-HER2 Assay Media [24] was replaced every 48 hours. Cultures were maintained for 9 days prior to photo-documentation. Digital images were taken in 4 random fields/well (3–4 spheroids/field). Total spheroid area was calculated using NIH Image J software. For spheroid proliferation analysis, cultures were fixed at day 5 in a 1:1 methanol: acetone solution for 10 minutes at −20°C and co-stained with anti-PCNA (1:1000) and nuclear stain TO-PRO3 (1:2000) as previously described [24]. Confocal images of 15 spheroids per experiment were taken at random and percentage of PCNA-positive nuclei was quantified. For AKT inhibitor-treated spheroid cultures, cells were allowed to grow for 48 hours, followed by daily AKT-inhibitor or vehicle control treatment starting on day3. For each analysis described above, at least two to three independent experiments were performed.

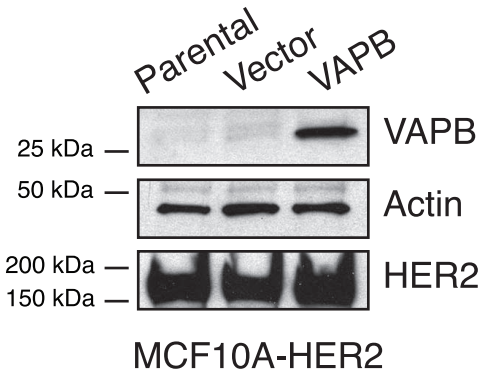
In vivo Tumor Studies

All animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. FVB female recipient animals were cleared of endogenous epithelium as described previously [26] and one million MMTV-Neu tumor cells were injected orthotopically. A total of 8–10 animals per group were analyzed in 2 independent experiments. Resulting tumors were harvested 4–5 weeks after injection for analysis of tumor volume (volume = length × width^2 × 0.52). Harvested tumors were further processed for H&E staining and immunohistochemistry analysis of proliferation (Ki67) or apoptosis (cleaved caspase-3). Proliferating tumor cells were quantified by enumerating Ki67 positive nuclei in 4 random fields per tumor and presented as a percentage of Ki67+nuclei/total nuclei. A total of 8–10 tumors were quantified in two independent experiments. Statistical significance was assessed by single factor ANOVA.

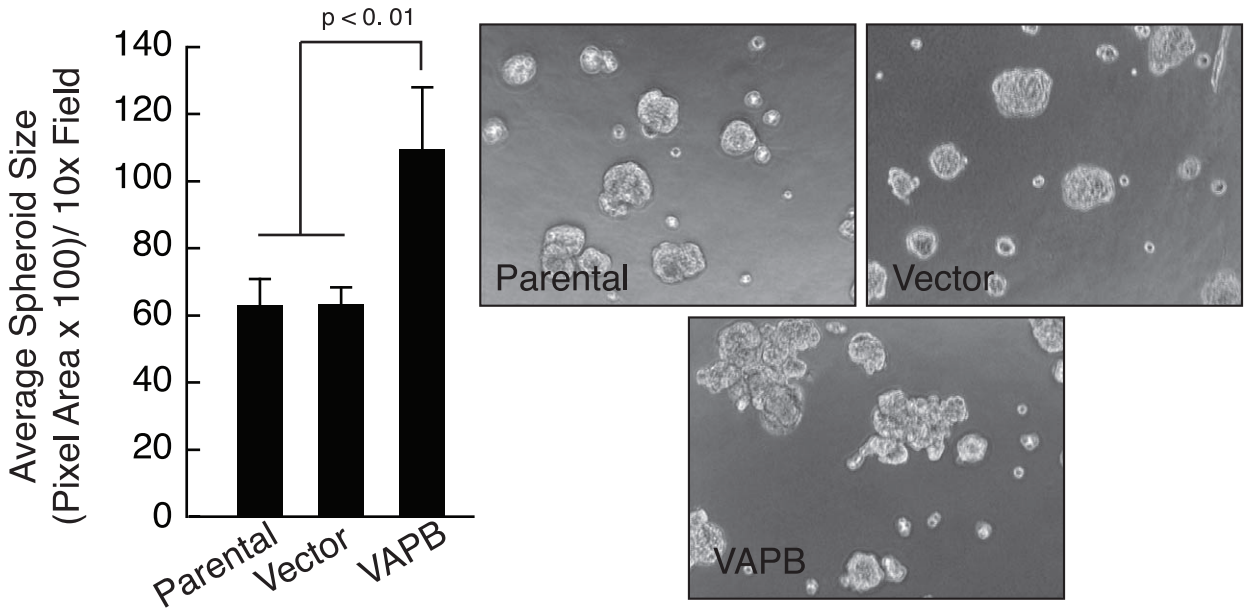
Immunoblotting

For immunoblot analysis of VAPB knockdown or overexpression, cells were washed with PBS and lysed in Radioimmunoprecipitation Assay (RIPA) buffer (50 mM Tris pH 7.4, 150 mM

A


Parental
Vector
VAPB
25 kDa
50 kDa
200 kDa
150 kDa
VAPB
Actin
HER2
MCF10A-HER2

B


p < 0. 01
Average Spheroid Size
(Pixel Area x 100)/ 10x Field
140
120
100
80
60
40
20
0
Parental
Vector
VAPB
Parental
Vector
VAPB

C

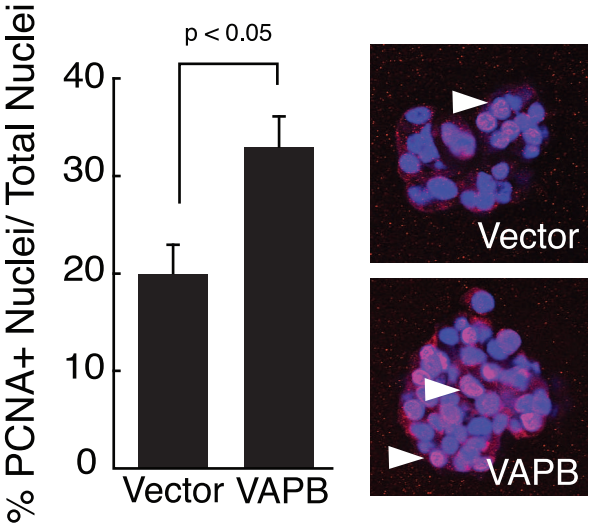

p < 0.05
% PCNA+ Nuclei/ Total Nuclei
40
30
20
10
0
Vector
VAPB
Vector
VAPB

Figure 2. VAPB expression enhances spheroid size and proliferation in mammary epithelial cells. (A) VAPB was stably expressed in MCF10A-HER2 cells via retroviral transduction, as judged by western blot analysis. (B) MCF10A-HER2-VAPB spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid ($p<0.01$, ANOVA). (C) Cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). Cell nuclei were stained blue by TO-PRO-3 nuclear stain. PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments ($p<0.05$, t test). Arrows: PCNA positive cells.
doi:10.1371/journal.pone.0046281.g002

NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitor tablets (Roche). Approximately 30–50 ug of total cell lysate was separated by SDS-PAGE and probed with anti-VAPB (1:1000) overnight in 5% nonfat dry milk/TBS-Tween (0.05%) and detected with anti-goat HRP (1:1000). For analysis of pAKT levels in VAPB expressing cells, cells were serum starved overnight and stimulated with DMEM/F-12 with 20 ng/mL of EGF for 20 minutes. For some experiments, an AKT inhibitor was added under normal growth conditions for 3 hours prior to cell lysis. Data are a representation of three independent experiments.

Statistical Analysis

Results are presented as mean values±standard error. *P*-values are given in the figure legends, and values of $P<0.05$ were considered to be statistically significant. Statistical analyses were performed by single factor ANOVA and unpaired, two-tailed Student *t*-test using PRISM software (GraphPad Sofware, La Jolla, CA, USA). For TMAs, statistical significance of VAPB expression between patient samples was determined by Chi-Square analysis.

Results

VAPB is Overexpressed in Breast Cancer and Negatively Correlates with Patient Survival

Several studies reported an increase in VAPB copy number or expression in breast cancer [13,14,27,28,29]. To determine the impact of VAPB expression on clinical outcome in human breast cancer, we analyzed VAPB expression in a published human breast cancer microarray dataset from a panel of 295 patient samples [19]. Kaplan-Meier analysis revealed that high levels of *VAPB* mRNA were significantly associated with poorer recurrence free survival (Figure 1A) than in patients with low *VAPB* mRNA expression. Similar results were obtained from a second, independent patient data set (n = 286) (Figure 1B) [20]. To assess VAPB protein expression in human breast cancer, we performed immunohistochemistry in breast cancer tissue microarrays (TMA, n = 84) with a previously validated anti-VAPB antibody [22]. As shown in Figure 1C, we observed a significant increase of VAPB expression in invasive ductal carcinoma and lymph node metastasis samples, compared to normal tissue ($p<0.01$, Chi-square test). Taken together these data highlight the clinical relevance of high VAPB expression in human breast cancer.

VAPB Promotes Tumor Spheroid Growth by Enhancing Tumor Cell Proliferation

Because the VAPB allele is often amplified in human breast cancer [13,14], including cancers that have HER2-amplification [27],we sought to determine if elevated VAPB expression enhances tumor cell growth. MCF10A-HER2 cells, an immortalized mammary epithelial cell line expressing the HER2 oncogene, were transduced to express human VAPB protein (Figure 2A). Tumor spheroid growth was quantified in a three-dimensional culture. As previously reported, control MCF10A-HER2 cells formed large acinar-like structures with a filled lumen [24]. Expression of VAPB in MCF10A-HER2 cells led to larger, irregular structures with invasive protrusions and a significant increase of spheroid size relative to parental and vector controls (Figure 2B). To determine whether increased tumor spheroid size is due to increased cell proliferation, the tumor spheroids were stained for PCNA, a cell proliferation marker. As shown in Figure 2C, VAPB expression elevated the number of PCNA-positive nuclei per spheroid, suggesting that VAPB regulates cell proliferation. Apoptosis, as measured by cleaved caspase 3 staining of 3D spheroid cultures, was not significantly changed (data not shown).

As an independent approach to determine whether VAPB is necessary for tumor cell growth, we knocked down VAPB in MMTV-Neu cells. These cells are derived from spontaneously arising mammary tumors from the MMTV-Neu mouse model [23]. Stable expression of two independent shRNAs significantly reduced VAPB protein levels (Fig. 3A) but not the related protein VAPA (Figure S1), suggesting that shRNAs specifically downregulated VAPB expression. In contrast to the morphology of vector control spheroids, which displayed disorganized and irregular structures with protrusions, knockdown of VAPB resulted in much smaller and more compact tumor spheroids. Since one of the VAPB knowndown (KD #1) targets the 3′ UTR, we were able to re-express full-length human VAPB protein (Figure 3B). KD cells with re-expression of VAPB restored tumor spheroid size and irregular morphology, demonstrating that phenotypes induced by shRNAs are VAPB specific and not due to off-target effects (Figure 3C). Consistent with the VAPB overexpression data, knockdown of VAPB significantly reduced proliferation in spheroid cultures as quantified by PCNA staining (Figure 3D). Together, these data indicate that VAPB regulates tumor spheroid size by increased tumor cell proliferation.

VAPB Promotes Tumor Growth in an Orthotopic Mammary Tumor Model

Next, we investigated the role of VAPB in tumor growth *in vivo* in a mammary tumor orthotopic transplantation model [15]. One million MMTV-Neu control or VAPB knockdown cells were injected into cleared mammary gland fat pads of FVB recipient female mice. VAPB knockdown tumor cells failed to form tumors or formed very small, non-palpable tumors at five weeks post-transplantation, compared to parental or vector controls (Figure 4A). While parental or vector control tumors display densely packed tumor cells, VAPB knockdown tumors exhibit a reduced mammary tumor cell content (Figure 4B). To examine changes within the tumor epithelium, we assessed tumor proliferation and apoptosis in tissue sections by staining for Ki67 and cleaved caspase-3, respectively. We observed a significant decrease in tumor cells with Ki67 nuclear staining (Figure 4C), whereas cleaved caspase-3 levels were unaffected (data not shown). These data suggest that loss of VAPB inhibits HER2-initiated mammary tumor proliferation *in vivo*.

VAPB-induced Cell Proliferation is Mediated by Elevated AKT Activity

To understand the mechanisms through which VAPB enhances proliferation, we investigated potential links between VAPB and

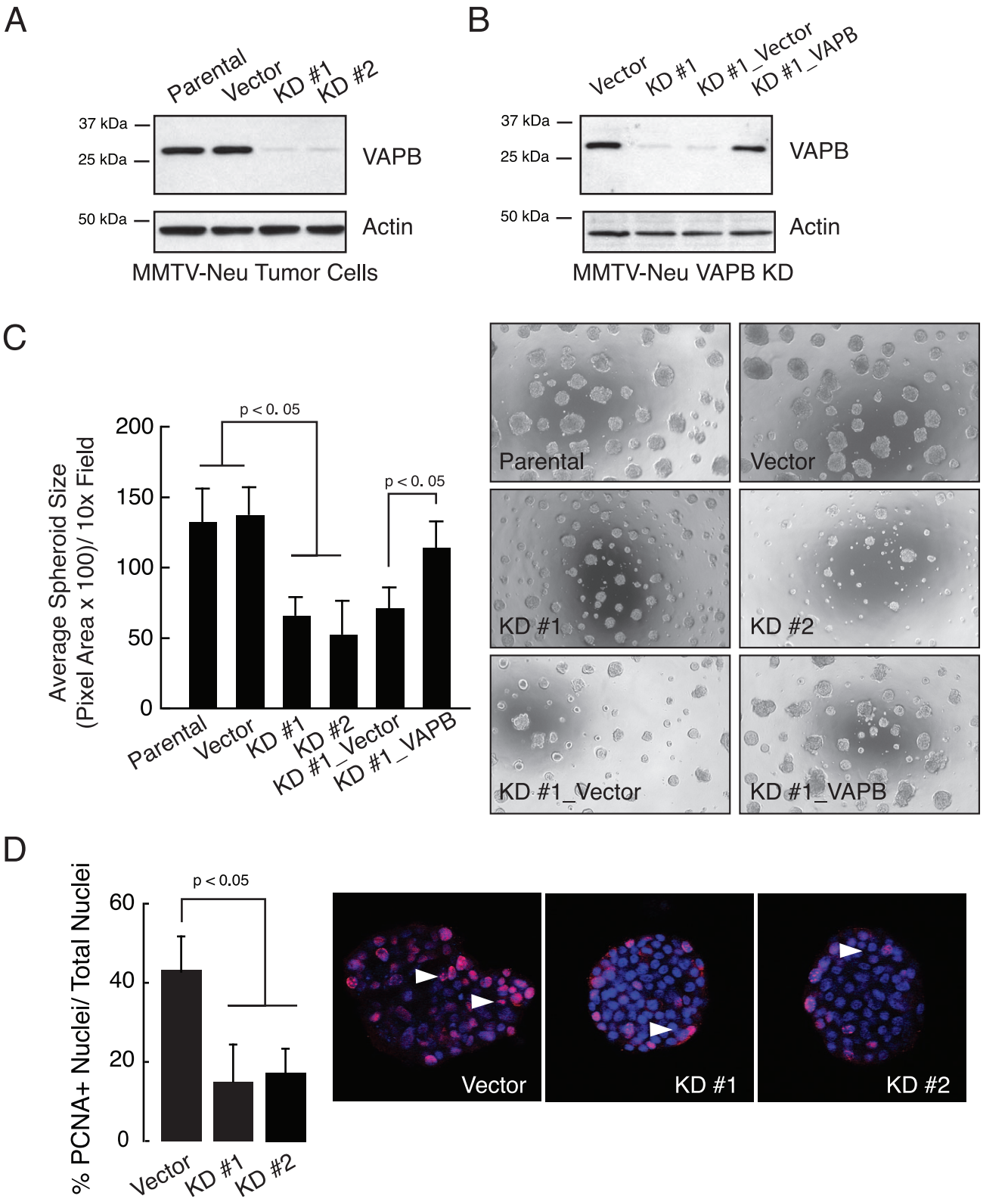


Figure 3. VAPB knockdown impairs mammary tumor spheroid growth and cell proliferation. (A) VAPB expression was silenced in MMTV-Neu tumor cells by two independent lentiviral-mediated shRNAs, as judged by western blot analysis. (B) VAPB knockdown (KD #1) cells were rescued by re-expressing a full-length human VAPB cDNA via retroviral transduction. (C) MMTV-Neu spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid ($p<0.01$, ANOVA). (D) MMTV-Neu cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments ($p<0.05$, t test). Arrows: PCNA positive cells.
doi:10.1371/journal.pone.0046281.g003

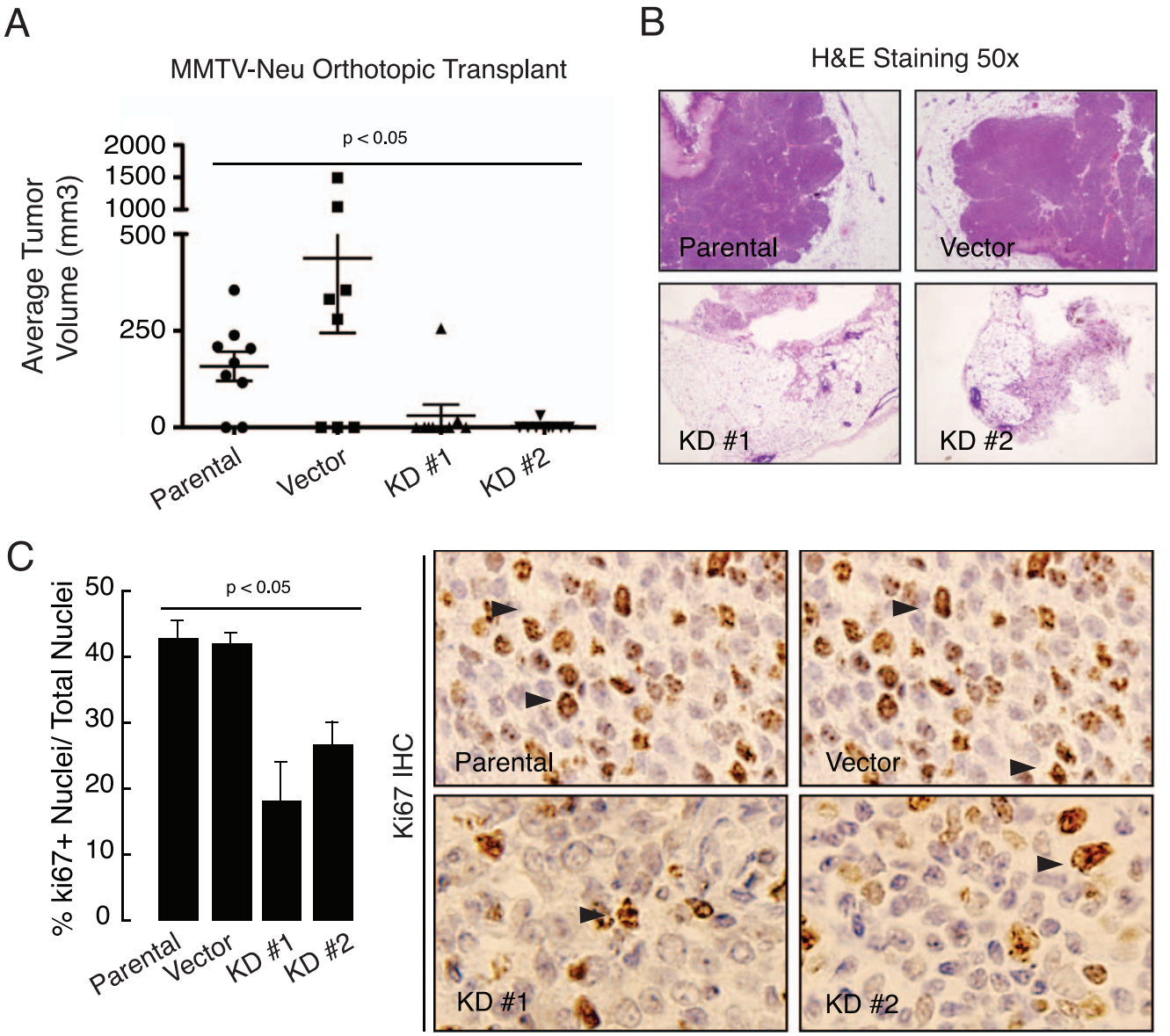
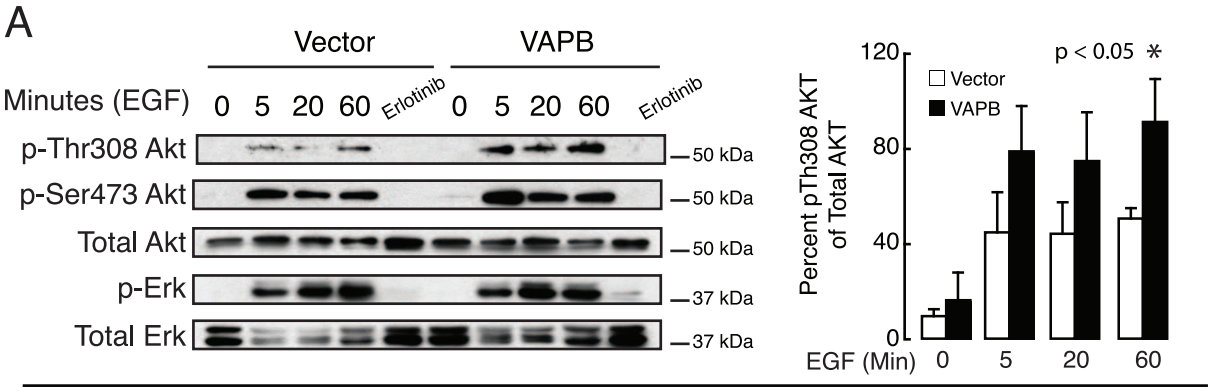
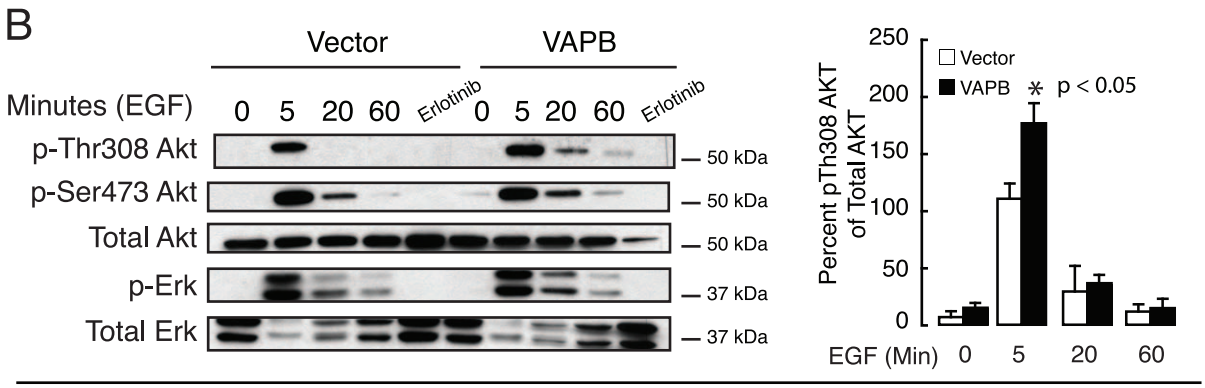


Figure 4. VAPB is required for tumor growth *in vivo*. (A) One million of MMTV-Neu control or VAPB knockdown cells were injected into cleared mammary gland fat pads of 3-week old FVB recipient female mice (n = 8−10/experimental condition). Tumors were harvested 5 weeks after transplantation. Tumor size was measured by a caliper and tumor volumes were calculated. ($p<0.05$, ANOVA). (B) Tumors were processed, sectioned and stained with H&E (B) or Ki67, a proliferation marker (C). Proliferating tumor cells were quantified by enumeration of Ki67+ nuclei and presented as a percentage of Ki67+ nuclei/total nuclei. VAPB knockdown cells showed a significant decrease in proliferation ($p<0.05$, ANOVA). Arrows: Ki67 positive cells.
doi:10.1371/journal.pone.0046281.g004

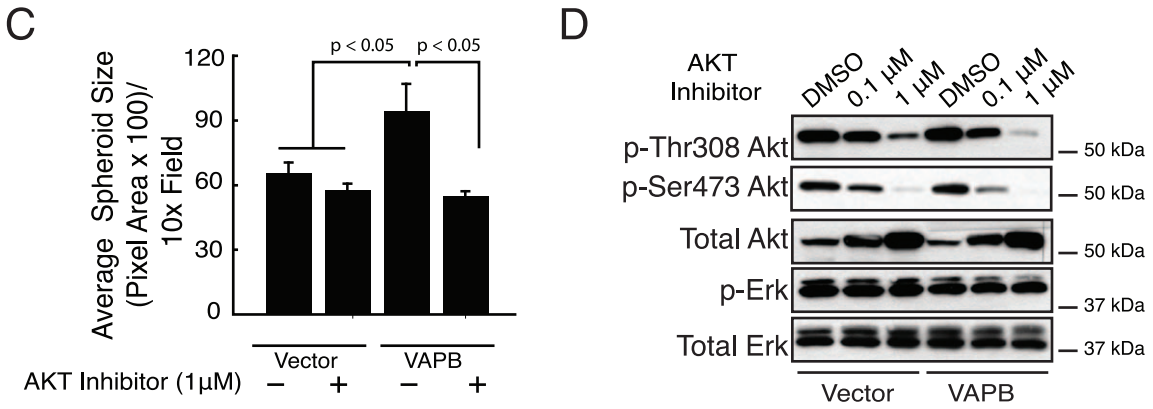
signaling molecules relevant to tumor growth. Because ERK and AKT signaling pathways are two major players in regulating cell proliferation in mammary tumor cells, we assessed their activities by western blot analysis. MCF-10A.HER2 cells carrying wild-type VAPB expression construct or the control vector were serum-starved overnight and stimulated with EGF at the indicated time points. In response to EGF stimulation, there is a rapid increase in phosphorylation of AKT at Thr308 and Ser473, indicating activation of its kinase activity. The phospho-AKT levels were more pronounced in cells overexpressing VAPB than those carrying the control vector (Figure 5A). In contrast, ERK phosphorylation levels were not changed between two cell populations. Likewise, knockdown of VAPB in MMTV-Neu cells reduced phospho-AKT levels upon EGF treatment without affecting phospho-ERK. Re-expression of VAPB in KD cells restored AKT phosphorylation to control levels, indicating that AKT may play a key role in regulating cell growth in these cells. To test if VAPB-mediated spheroid growth is dependent on AKT phosphorylation, cells were treated with an allosteric AKT inhibitor [18]. AKT inhibition significantly reduced spheroid growth of VAPB expressing cells (Figure 5B and 5E). As expected the AKT inhibitor reduced phosphorylated AKT, while phospho-ERK was unaffected (Figure 5C and 5F). Although these results do not rule out the contribution of other signaling pathways, our findings suggest that VAPB-induced proliferation is mediated, at least in part, through activation of the AKT pathway.



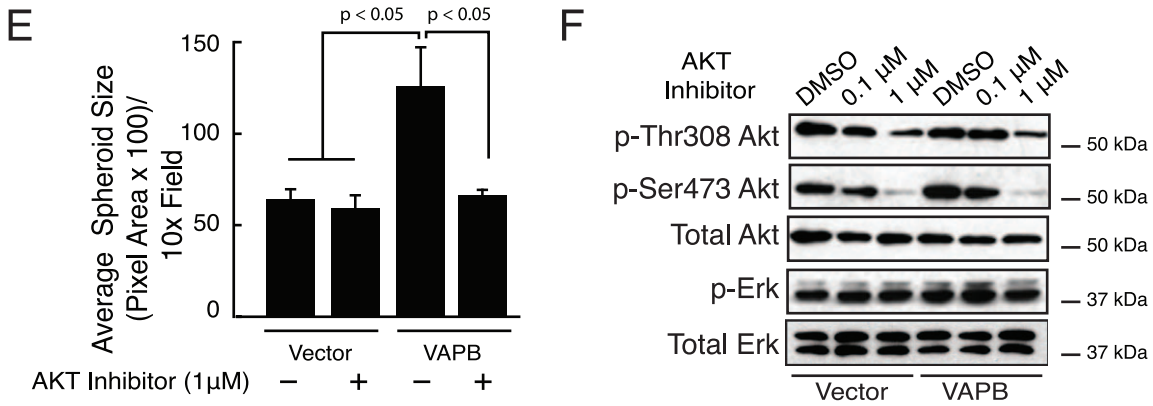
MCF10A-HER2 Cells



MMTV-Neu VAPB Knockdown Cells



MCF10A-HER2 Cells



MMTV-Neu VAPB Knockdown Cells

Figure 5. VAPB-dependent cell growth is mediated through AKT activity. (A) MCF-10A cells expressing VAPB or carrying control vector were serum starved and stimulated with 20 ng/mL EGF at the indicated time points. Phospho-AKT levels in MCF10A-HER2 cells were measured by western blot analysis. (B) VAPB was knocked down in MMTV-Neu cells (KD#1) and re-expressed via retroviral transduction (KD#1_VAPB). Phospho-AKT levels were measured by western blot analysis Representative blots from 3 independent experiments are shown. (C–E) Pharmacologic AKT inhibition significantly impaired VAPB-mediated spheroid growth in MCF10A-Her2 cells (C) and MMTV-Neu VAPB knockdown cells rescued with VAPB reexpression (E) ($p<0.05$ ANOVA). Inhibition of AKT activity was confirmed by western blot analysis for phospho-AKT in MCF10A-HER2 cells (D) and MMTV-Neu knockdown cells (F).
doi:10.1371/journal.pone.0046281.g005

Discussion

VAPB was originally identified as one of the vesicle-associated membrane protein (VAMP) associated proteins. Although VAPB was implicated in a wide range of cellular processes, its function in cancer has not been characterized. In this report, we provide evidence that VAPB regulates mammary tumor growth and proliferation via activation of AKT activity. VAPB protein expression is elevated in primary and metastatic tumor specimens, and VAPB mRNA expression levels correlated negatively with patient survival in two large breast tumor datasets. Overexpression of VAPB increased spheroid size and proliferation in MCF10A-HER2 cells. Conversely, knockdown of VAPB in MMTV-Neu mammary tumor cells inhibited tumor cell proliferation in 3-D culture *in vitro* and suppressed tumor growth in orthotopic mammary tumor allografts. The growth regulation of mammary tumor cells controlled by VAPB appears to be mediated, at least in part, by modulation of AKT activities. Collectively, the genetic, functional and mechanistic analyses suggest a role of VAPB in tumor promotion in human breast cancer.

In breast cancer, approximately 30% of tumors have mutations in one or more components of the PI3K/AKT pathway [30]. Two lines of evidence suggest that VAPB expression modulates AKT activation. First, overexpression of VAPB in MCF10A-HER2 cells enhances phosphorylation of AKT, whereas knockdown of VAPB in MMTV-Neu tumor cells inhibited pAKT levels. Furthermore, the addition of an allosteric AKT inhibitor significantly reduced 3D spheroid growth induced by VAPB. It is currently unclear how VAPB regulates AKT activity. Since VAPB does not contain known enzymatic activities, it is likely that its action is mediated by its interaction with other proteins.

Major subcellular compartments where VAPB is localized are the ER and Golgi, where secretory and membrane proteins are synthesized and transported to cell surface [3,4]. Several studies show that VAPB is required for neurotransmitter release and functions in early secretory transport events [5,7]. Therefore it is possible that VAPB may regulate the protein secretion. Indeed, when VAPB is overexpressed in MCF10A-HER2 cells, transport of VSV-G-GFP ts045, a protein used to monitor vesicle traffic from ER/Golgi to the plasma membrane, is markedly enhanced (Figure S2), consistent with a previous report that show VAPB deficiency inhibited VSV-G-GFP ts045 to the plasma membrane in HeLa cells [31]. Accordingly, one mechanism by which VAPB promotes tumor cell proliferation could be through secretion of growth factors, cytokines, matrix metalloproteinases, as well as delivery of receptors to the cell surface [17]. This possibility is further strengthened by the fact that VAPB physically interacts with Arf1 and Rab1 (Figure S3), two small GTPases that are known to play critical roles in regulation of vesicle trafficking in the secretary pathway [32,33,34,35,36]. Interestingly, Arf1 has also been implicated in recruitment of p85 subunits of PI3K to EGFR in breast cancer cells [37], providing an additional possible mechanism by which VAPB could regulate AKT activities.

Aside from interacting with Arf1/Rab1, we and others also found VAPB in complex with other proteins including lipid transfer binding proteins such as Nir2 (Table S1) [22,31], a phosphoinositol/phosphotidyalcholine transfer protein. Deletion of *scs2*, the yeast homolog of VAPs, was reported to reduce phosphoinosotide levels [38]. Since AKT phosphorylation is dependent on recruitment to the plasma membrane through interaction between the PH domain and PIP3, such alterations in phosphoinositols at the plasma membrane could affect ultimately the activation of AKT. Given the diverse function of VAPB-interacting proteins (Table S1), it is likely that multiple pathways converge through VAPB to enhance the AKT pathway and affect tumor cell proliferation.

Our results suggest that VAPB enhances breast tumor cell proliferation is mediated through the AKT pathway. However we cannot rule out other functions of VAPB that also may contribute to the phenotypes observed in this study, such as lipid sensing and transport [39,40,41] ER and Golgi architecture [22,31], and the unfolded protein response [6,10]. Therefore further studies are needed to address whether perturbation in these mechanisms alters the phenotypes of tumor cells in relation to VAPB expression. Furthermore, although full-length VAPB localizes to the ER, Golgi, and membrane-bound vesicles [3,42], a number of studies showed that the MSP domain of VAPB can be secreted and act as an antagonist for Eph receptor tyrosine kinases in *C. elegans* and *Drosophila* [9,43]. Secreted MSP has also been detected in human serum [9]. Because ligand-dependent EphA2 receptor signaling has been associated with tumor suppression whereas EphA2 ligand-independent signaling promotes tumor initiation and malignancy in breast cancer [44,45], it is tempting to speculate that the secreted MSP domain may compete with ligand for binding to EphA2 receptor, thereby blocking EphA2-tumor suppressive function. However, although tumor cells do secrete MSP (Rao and Chen, unpublished data), further investigations are required to test this hypothesis.

In summary, we identified a functional role of VAPB in promoting tumor cell proliferation in breast cancer. This discovery opens up a number of exciting avenues for future studies of both full length VAPB and the secreted MSP domain in cancer. As VAPB overexpression is associated with poor patient survival, targeting VAPB-associated protein secretory pathway may provide novel targets for future pharmacological strategies in cancer therapy.

Supporting Information

Figure S1 Knockdown of VAPB does not affect VAPA expression. VAPB expression was silenced in MMTV-Neu tumor cells by two independent lentiviral-mediated shRNAs (Figure 3A). VAPA protein expression was not affected by VAPB shRNA, as judged by western blot analysis, demonstrating the specificity of VAPB shRNAs. (Materials and Methods S1).
(EPS)

Figure S2 VAPB expression does not affect apoptosis. (A) MCF10A-HER2 or (B) MMTV-Neu knockdown cells were stained for cleaved caspase-3 at day 8 in 3-dimensional culture. Cleaved caspase-3 positive spheroids (green) were quantified by confocal microscopy analysis. No significant changes were

observed in both cell lines. (C) in vivo analysis of apoptosis was measured by cleaved caspase-3 staining of tumor sections. Cleaved caspase-3 positive nuclei were quantified. VAPB deficiency does not significantly affect apoptosis in vivo. Arrows: cleaved caspase-3 positive cells. (Materials and Methods S1).
(EPS)

Figure S3 PCNA analysis of tumor allografts. Proliferating tumor cells were quantified by enumeration of PCNA- positive nuclei and presented as a percentage of PCNA+ nuclei/total nuclei. VAPB knockdown tumors showed a significant decrease in proliferation ($p<0.05$, ANOVA). Arrows: PCNA positive cells. (Materials and Methods S1).
(EPS)

Figure S4 Analysis of AKT and ERK activities in VAPB expressing cells. MCF10A-HER2 (A) and MMTV-Neu knockdown cells (B) expressing VAPB or carrying control vector were serum starved and stimulated with 20 ng/mL EGF at the indicated time points. Phospho-ERK levels were measured by western blot analysis and quantified. (C) VAPB was knocked down in MMTV-Neu cells (KD#1) and re-expressed via retroviral transduction (KD#1_VAPB). Phospho-AKT and phospho-ERK levels were measured by western blot analysis. Representative blots from 3 independent experiments are shown. (Materials and Methods S1).
(EPS)

Figure S5 PI3K inhibition attenuates VAPB dependent spheroid growth. (A) Cells were cultured in 3D Matrigel for 2 days and then treated with LY294002 (20 μM) or vehicle control every 2 days. Tumor cell spheroids were quantified at day 8. (B) Inhibition of AKT activity was confirmed by western blot analysis for phospho-AKT levels. Shown were representative blots from two independent experiments. (Materials and Methods S1).
(EPS)

Figure S6 VAPB facilitates the transport of secretory proteins to cell surface. (A) MCF10A-HER2-VAPB expressing cells were transfected with the ts045 temperature sensitive vesicular stomatitis viral glycoprotein (VSVG) GFP and incubated at 40°C for 16 hours to accumulate misfolded VSVG protein in the ER. Following a 30-minute incubation with cyclohexamide, the cells were shifted to a permissive temperature (32°C) to allow transport along the secretory pathway. Total VSVG was visualized by GFP fluorescence (green) and cell surface VSVG was detected using an antibody against VSVG ectodomain (red) under non-permeable condition. (B) The kinetics of appearance of VSVG-GFP at the cell surface was measured by cell-surface biotinylation and subsequent quantification of immnoblots with anti-VSVG in two independent experiments ($p<0.05$, unpaired t test). (Materials and Methods S1).
(EPS)

Figure S7 VAPB interacts with Arf1 and Rab1 small GTPases. HEK 293T cells were co-transfected with VAPB-Myc and Arf1-HA or FLAG-Rab1 expression constructs. VAPB and Arf1 or Rab1 was immunoprecipitated from cell lysates by anti-Myc, anti- HA, or anti-FLAG, and the resulting protein complexes were analyzed by western blot for Arf1 (A) and Rab1b (B) or VAPB, respectively. (Materials and Methods S1).
(EPS)

Table S1 Representative Candidates of VAPB binding proteins. MMTV-Neu control or VAPB knockdown cells were treated with chemical crosslinkers prior to lysis. Cell lysates were immunoprecipitated with anti-VAPB and resulting protein complexes were subjected to mass spectrometry analysis. The following criteria were used for selection of candidate proteins: (1) spectral counts ≥5 and (2) the ratio of vector/knockdown ≥4. 170 candidate proteins were classified based on biological processes as annotated in PANTHER [5]. Selected functional groups and proteins are listed in TableS1.
(EPS)

Materials and Methods S1.
(DOC)

Acknowledgments

We acknowledge the Vanderbilt Mass Spectrometry Research Center and Dr. Hayes McDonald for support on sample analyses and data interpretations and the Vanderbilt Translational Pathology Shared Resource for tumor section immunohistochemistry. Confocal microscopy was performed in part through the use of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126). We also thank Mr. Yoonha Hwang for helping in generating some of the constructs. Temperature-sensitive VSVG-GFP construct was generously provided by Dr. Jennifer Lippencott-Swartz (National Institute of Health).

Author Contributions

Conceived and designed the experiments: MR JC. Performed the experiments: MR WS DBS AJ. Analyzed the data: MR. Contributed reagents/materials/analysis tools: DG SL YS. Wrote the paper: MR JC.

References

1. Nishimura Y, Hayashi M, Inada H, Tanaka T (1999) Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. Biochem Biophys Res Commun 254: 21–26.
2. Weir ML, Klip A, Trimble WS (1998) Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. Biochem J 333 (Pt 2): 247–251.
3. Moumen A, Virard I, Raoul C (2011) Accumulation of wildtype and ALS-linked mutated VAPB impairs activity of the proteasome. PLoS One 6: e26066.
4. Skehel PA, Fabian-Fine R, Kandel ER (2000) Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. Proc Natl Acad Sci U S A 97: 1101–1106.
5. Soussan L, Burakov D, Daniels MP, Toister-Achituv M, Porat A, et al. (1999) ERG30, a VAP-33-related protein, functions in protein transport mediated by COPI vesicles. J Cell Biol 146: 301–311.
6. Gkogkas C, Middleton S, Kremer AM, Wardrope C, Hannah M, et al. (2008) VAPB interacts with and modulates the activity of ATF6. Hum Mol Genet 17: 1517–1526.
7. Skehel PA, Martin KC, Kandel ER, Bartsch D (1995) A VAMP-binding protein from Aplysia required for neurotransmitter release. Science 269: 1580–1583.
8. Loewen CJ, Roy A, Levine TP (2003) A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. EMBO J 22: 2025–2035.
9. Tsuda H, Han SM, Yang Y, Tong C, Lin YQ, et al. (2008) The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. Cell 133: 963–977.
10. Kanekura K, Nishimoto I, Aiso S, Matsuoka M (2006) Characterization of amyotrophic lateral sclerosis-linked P56S mutation of vesicle-associated membrane protein-associated protein B (VAPB/ALS8). J Biol Chem 281: 30223–30233.
11. Nishimura AL, Mitne-Neto M, Silva HC, Richieri-Costa A, Middleton S, et al. (2004) A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. Am J Hum Genet 75: 822–831.
12. Suzuki H, Kanekura K, Levine TP, Kohno K, Olkkonen VM, et al. (2009) ALS-linked P56S-VAPB, an aggregated loss-of-function mutant of VAPB, predisposes motor neurons to ER stress-related death by inducing aggregation of co-expressed wild-type VAPB. J Neurochem 108: 973–985.

13. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, et al. (2006) Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 10: 529–541.
14. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, et al. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515–527.
15. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, et al. (2008) The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. J Clin Invest 118: 64–78.
16. Chen J (2012) Regulation of tumor initiation and metastatic progression by eph receptor tyrosine kinases. Adv Cancer Res 114: 1–20.
17. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646–674.
18. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, et al. (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. Bioorg Med Chem Lett 15: 761–764.
19. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AAM, et al. (2002) A gene-expression signature as a predictor of survival in breast cancer. New England Journal of Medicine 347: 1999–2009.
20. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, et al. (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 365: 671–679.
21. Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, et al. (2002) Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 21: 7011–7026.
22. Amarilio R, Ramachandran S, Sabanay H, Lev S (2005) Differential regulation of endoplasmic reticulum structure through VAP-Nir protein interaction. J Biol Chem 280: 5934–5944.
23. Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, et al. (2003) Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. Mol Cell Biol 23: 8691–8703.
24. Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256–268.
25. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, et al. (2004) Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. J Biol Chem 279: 24505–24513.
26. Brantley DM, Chen CL, Muraoka RS, Bushdid PB, Bradberry JL, et al. (2001) Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium. Mol Biol Cell 12: 1445–1455.
27. Arriola E, Marchio C, Tan DS, Drury SC, Lambros MB, et al. (2008) Genomic analysis of the HER2/TOP2A amplicon in breast cancer and breast cancer cell lines. Lab Invest 88: 491–503.
28. Rizki A, Weaver VM, Lee SY, Rozenberg GI, Chin K, et al. (2008) A human breast cell model of preinvasive to invasive transition. Cancer Res 68: 1378–1387.
29. Edgren H, Murumagi A, Kangaspeska S, Nicorici D, Hongisto V, et al. (2011) Identification of fusion genes in breast cancer by paired-end RNA-sequencing. Genome Biol 12: R6.
30. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, et al. (2008) An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 68: 6084–6091.
31. Peretti D, Dahan N, Shimoni E, Hirschberg K, Lev S (2008) Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport. Mol Biol Cell 19: 3871–3884.
32. D'Souza-Schorey C, Chavrier P (2006) ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 7: 347–358.
33. Gillingham AK, Munro S (2007) The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol 23: 579–611.
34. Spang A (2002) ARF1 regulatory factors and COPI vesicle formation. Curr Opin Cell Biol 14: 423–427.
35. Plutner H, Cox AD, Pind S, Khosravi-Far R, Bourne JR, et al. (1991) Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. J Cell Biol 115: 31–43.
36. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.
37. Boulay PL, Cotton M, Melancon P, Claing A (2008) ADP-ribosylation factor 1 controls the activation of the phosphatidylinositol 3-kinase pathway to regulate epidermal growth factor-dependent growth and migration of breast cancer cells. J Biol Chem 283: 36425–36434.
38. Kagiwada S, Zen R (2003) Role of the yeast VAP homolog, Scs2p, in INO1 expression and phospholipid metabolism. J Biochem 133: 515–522.
39. Jansen M, Ohsaki Y, Rita Rega L, Bittman R, Olkkonen VM, et al. (2011) Role of ORPs in sterol transport from plasma membrane to ER and lipid droplets in mammalian cells. Traffic 12: 218–231.
40. Kawano M, Kumagai K, Nishijima M, Hanada K (2006) Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT. J Biol Chem 281: 30279–30288.
41. Loewen CJ, Levine TP (2005) A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. J Biol Chem 280: 14097–14104.
42. Lapierre LA, Tuma PL, Navarre J, Goldenring JR, Anderson JM (1999) VAP-33 localizes to both an intracellular vesicle population and with occludin at the tight junction. Journal of Cell Science 112: 3723–3732.
43. Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D (2003) An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in Caenorhabditis elegans. Genes Dev 17: 187–200.
44. Chen J, Zhuang G, Frieden L, Debinski W (2008) Eph receptors and Ephrins in cancer: common themes and controversies. Cancer Res 68: 10031–10033.
45. Vaught D, Brantley-Sieders DM, Chen J (2008) Eph receptors in breast cancer: roles in tumor promotion and tumor suppression. Breast Cancer Res 10: 217.